The Ins and Outs of Bioaccumulation

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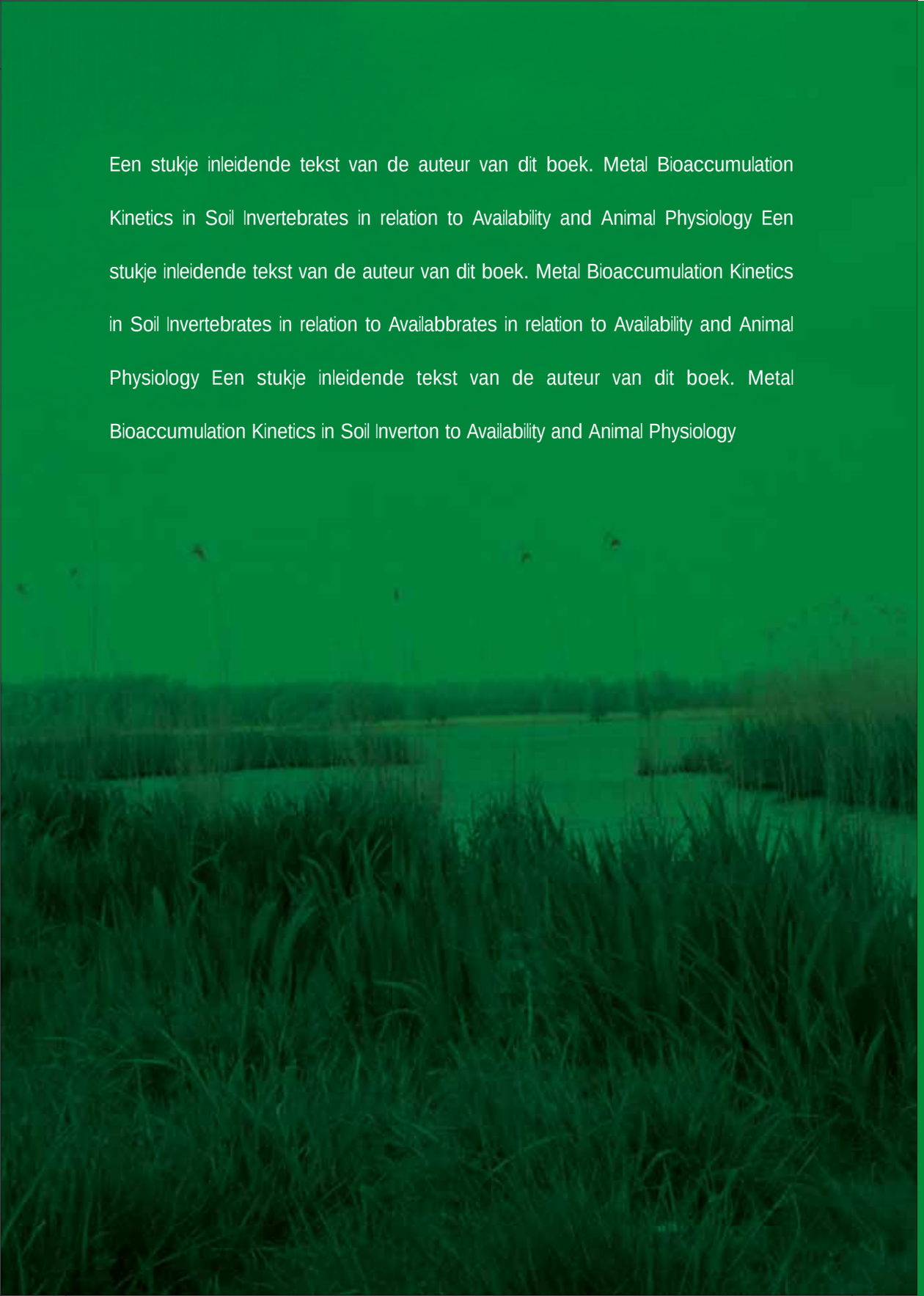
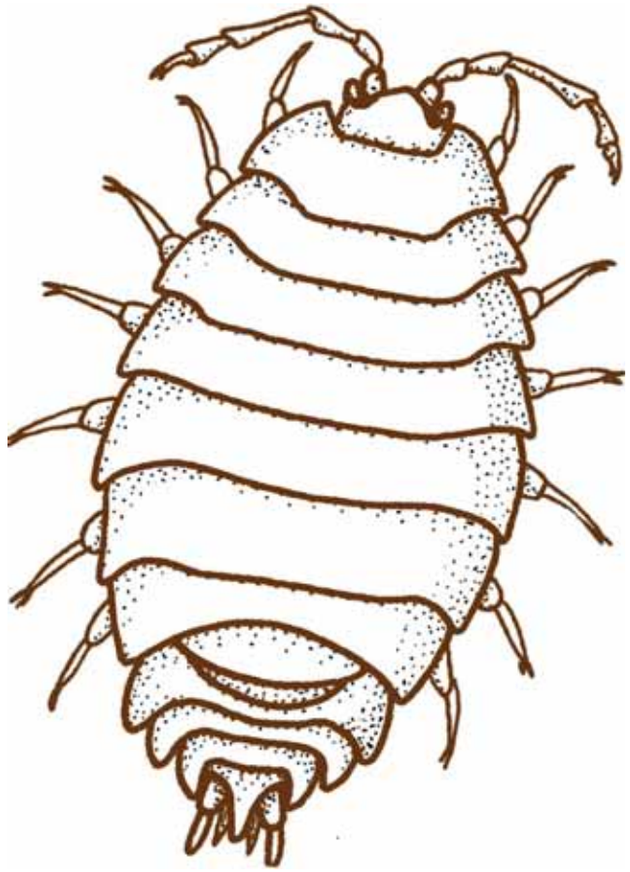
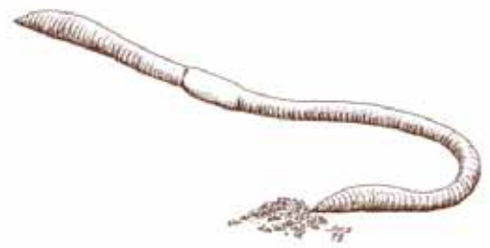


The ins and outs of bioaccumulation Martina G. Vijver

The ins and outs of bioaccumulation



Metal Bioaccumulation Kinetics in Soil Invertebrates in Relation to Availability and Animal Physiology



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VRIJE UNIVERSITEIT

The Ins and Outs of Bioaccumulation

Metal Bioaccumulation Kinetics in Soil Invertebrates in Relation to Availability and Physiology

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The Ins and Outs of Bioaccumulation

Metal Bioaccumulation Kinetics in Soil Invertebrates in Relation to Availability and Physiology

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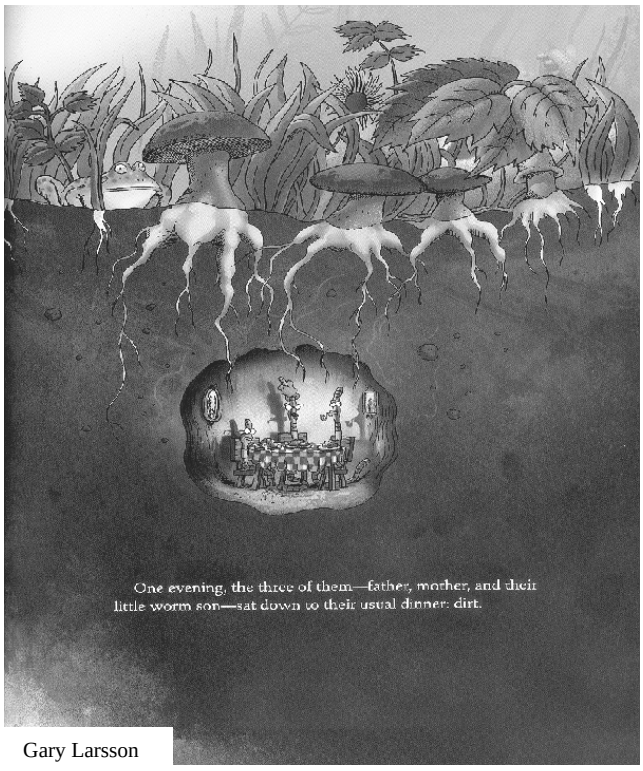

Gary Larsson

Chapter 1
General introduction

1.1 Metals in floodplain soils

Floodplains are wonderful ecosystems, in which the dynamic influences of a river interact with a partly terrestrial, partly aquatic environment. In these ecosystems a host of gradients in elevation, soil texture and vegetation can be found, resulting in a large biodiversity. The canalisation of rivers, deforestation and urbanisation in the 1950s in The Netherlands had significantly affected the dynamic environment and the associated biodiversity, and moreover impaired the system's capacity to retain water. Redevelopment of floodplain systems is the new policy of the Dutch Ministry of Transport, Public Works and Water Management (Rijkswaterstaat). The policy aims to restore natural situations with better water retention over a longer period by increasing the meandering character of rivers, thereby creating water retention basins and returning the dynamics in floodplains. The gradients of the natural landscape will return as well as the flora and fauna. To achieve this redevelopment in floodplain systems, side channels are dug thereby often uncovering polluted soil layers. As a gift from the past, many Dutch floodplain soils are loaded with high concentrations of heavy metals and other contaminants. Local contamination of floodplain sites with metals already started around 1900. Water of rivers became especially seriously polluted in 1970s, due to the impact of human activities, such as extensive industrialisation and mining activities (Beurskens et al. 1993). Political and societal awareness of the serious pollution in the 1980s led to control measurements and, as a result, metal concentrations in the rivers decreased considerably (De Boo and Middelkoop 1999). Nevertheless metal pollution had affected the riverbeds and floodplains where contaminated sediments have been deposited in recent decades. As a consequence, nowadays elevated metal levels are detected in organisms inhabiting these floodplain soils (Hobbelen et al. 2004, Van der Scheer and Gerritsen 1998) and it is not unlikely that this biota is at potential risk (Vink and Hendriks 1999). As large parts of the floodplains will function as nature reserves, it is important to get insight into the ecological risks of the current and future pollution.

The mechanisms behind metal accumulation from soil in floodplain inhabiting biota are not yet unraveled. In general, availability of metals is widely accepted as one of the key factors governing bioaccumulation and, therefore, the potential environmental hazard to biota. However, many additional factors, correlated to each other, have an impact on the possible risks of metals. For instance, under field conditions mostly long-term chronic exposure of biota to low concentrations of metals occurs and mechanisms such as tolerance and adaptation play a role. Furthermore, floodplain systems are very dynamic fields, continuously subjected to inundation and desiccation. These dynamic processes have a major impact on the chemical and biological processes affecting bioaccumulation. Inundation affects many environmental factors, such as the chemical partitioning of metals over the different phases, the reduction-oxidation conditions of the soil, and leaf litter present as a food source for many soil-dwelling organisms. These environmental conditions all influence the behaviour of organisms, which

subsequently results in different internal metal concentrations. The interactions between chemistry and biology, driven by the dynamic character of the flood plains, make this ecosystem interesting and difficult to study. Unravelling underlying mechanisms of bioaccumulation may assist in the understanding of mechanisms behind metal uptake and biotic response, to allow for an accurate assessment before reconstructing river systems. The Institute for Inland Water management and Waste Water Treatment (RIZA) initiated the question to derive a scientifically based transfer function between metal speciation in flood plain soils subjected to changes and the uptake and possible ecological risks that metals can cause in soil invertebrates. The challenge of this PhD-research was to create more insight into metal uptake mechanisms by soil invertebrates and create a link to physico-chemical metal availability. Unique is the attempt to link information on metal availability to the physiology of soil invertebrates.

1.2 Defining bioavailability of metals

Results from daily practice show that a certain level of metal contamination can be toxic and present a significant risk, while at the same total level but under different conditions, metal contamination may prove to be harmless (Sauvé et al. 2000). This shows that bioavailability cannot simply be defined in one term, because it is a composite concept. To create an unequivocal understanding of metal bioavailability, it is separated in three specified processes (Dickson et al. 1994): chemical, biological and toxicological availability.

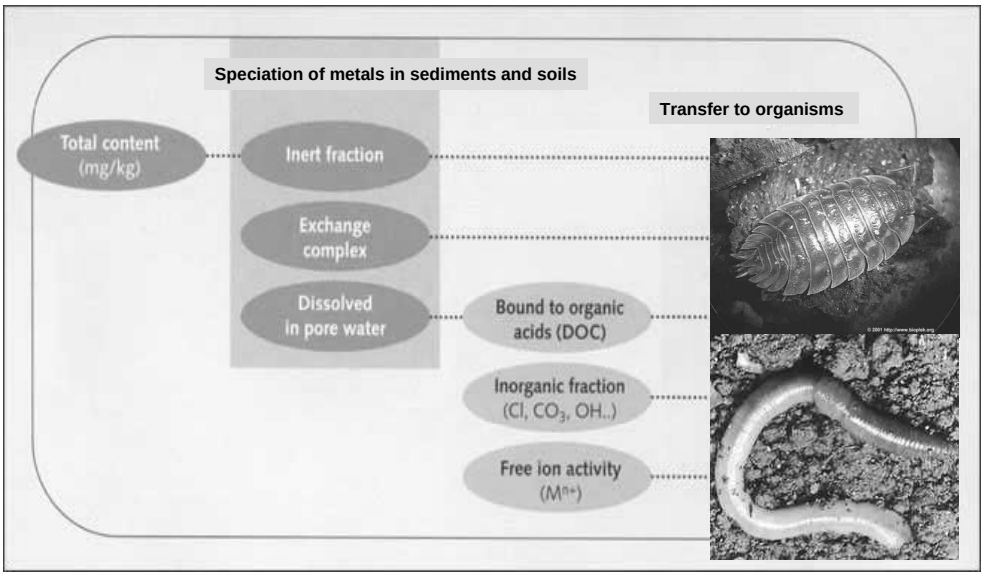

Figure 1: Schematic view of metal speciation in soils and sediments. Total metal content can be divided over the various fractions shown. Lines are drawn from all metal fractions towards the organisms because the transfer towards animals is organism-specific and up-to-now no consistent relationship has been found between any metal species and bioaccumulation (modified from Vink, www.riza.nl/sofie).

Chemical availability

Chemical availability is a physico-chemically driven supply process. The impact of metals on the environment is closely related to the form in which the metal occurs, so-called metal speciation. By speciation of a metal in soil, the total content of the metal is partitioned over

both the solid and the solution phase. In the solid phase, metals exist as precipitates (e.g. ferro sulfides and crystalline FeS, FeS_2 and Fe_3S_4, manganese sulfides), adsorbed on reactive soil surfaces and occluded or bound into soil minerals. Metals in the soil solution can exist as free metal ion or as species bound to inorganic and organic complexes. Figure 1 represents a schematic overview of the different components of metal speciation.

The metal concentration in the solution phase is regulated by adsorption; in a more or less descending order of affinity (Wijdeveld and Smits 1997) metals are bound to:

- Organic matter (particulate and dissolved humic substances having many active carboxylic and phenolic hydroxyl groups, highly depending on the origin and history of the organic matter (Senesi 1992).
- Hydrous iron oxides (Fe(OOH), amorphous goethite).
- Oxides of manganese (MnO_2, amorphous birnessite).
- Aluminium hydroxide and silicates ($Al(OH)_3$, amorphous, gibbsite, $Al(SiO_2)_3$, amorphous allophanes).
- Clay minerals (e.g. the dominant clay minerals in many Dutch soils are illite, kaolinite, vermiculite and bentonite. The metal-sorption capacity of clay minerals varies among the various minerals and is based on the density of negative charges on the clay surface)
- Calcium carbonate (amorphous, calcite)

Environmental conditions control the sorption of metals. One of the major factors influencing metal speciation is the acidity (pH) of the soil. An increase in pH changes the solid-solution partitioning of cations towards the solid phase, and hence the mobility and availability of cations generally decreases. Another controlling factor is the available amount of sorption sites related to the cation-exchange capacity of a soil and the amount of competitively sorbed cations, especially calcium. When many cations compete for the same sorption sites, the solid-solution partitioning of cations shifts towards the solution phase, and hence the mobility and availability of cations generally increases. Metal partitioning over the different sorption phases is assumed to be in equilibrium. Depletion of one of the metal species may occur, mostly the free metal ion activity because it reflects the chemical reactivity of a metal and is rapidly taken up by organisms. Disequilibrium due to depletion may lead to a new equilibrium situation. The time in which a new equilibrium is established depends on the total dissolved metal concentration and the concentration and nature of the ligands present in the solution. The release of free ion (Me^{2+} or Me^{2+} (H_2O)), replenished from metals bound to inorganic ligands in the solution (e.g. MeOH, $MeCl_2$), requires only seconds, whereas the release of metals from the solid phase towards the solution phase takes days rather than hours. In conclusion, total metal concentrations in soil include non-bioavailable forms. All loosely-bound metal species in the soil are in equilibrium with each other, however equilibration time depends on the nature of the ligand present. Metals in the solution phase reflect the species being chemically most available and are likely to be of most importance for uptake by organisms. However up to now, no consistent relationship has been found between any metal species and either bioaccumulation or toxicity.

Biological availability

Biological availability is a physiologically driven uptake process. The biologically available metal fraction is the fraction actually taken up by organisms. The magnitude of metal accumulation depends on the metal supply of the soil and the demand of soil organisms for metal uptake. Which metal species in the soil can be taken up by organisms is organism-specific and influenced by the possible uptake routes. Quantification of exposure routes of metals for terrestrial invertebrates is difficult and cannot yet be done accurately (Belfroid and Van Gestel 1999). From an ecological point of view, two metal uptake routes, or a combination of them, may be envisaged for soil invertebrates. Firstly, metal uptake via the permeable external surfaces was distinguished. The direct source of importance of this uptake route is metals in pore water. Secondly, metal uptake via the oral route was distinguished. Metal uptake via the oral route includes possible modification of metal speciation by gut conditions. This route includes uptake via drinking, soil and food ingestion. Via drinking, metal uptake is indirectly from pore water, possibly following modification of the pore water depending on gut conditions. Upon soil and food ingestion the metal uptake is indirectly from solid phases, possibly following modification by gut conditions. When focusing on bioaccumulation, problems around metal availability (such as which species are chemically reactive and how fast is metal replenishment) and metal bioavailability (such as which metal species are bioavailable, the contribution of uptake routes, and the physiological need for essential metals) are integrated and overcome.

The entry of metals into organisms is described by kinetic models, derived from linear compartment theory. Such models are used extensively in this thesis.

Toxicological availability

Toxicological availability is the toxico-dynamic behaviour of metals inside organisms. Accumulated metals are distributed throughout the organism's body, and can be sequestrated in a biologically available form or isolated metabolically to be no longer toxicologically available. At cellular level, the internal metal speciation which can be found in an organism is (Vijver et al. 2004):

- free ionic form or complexed ion species (e.g. $CdCl_2$, $CdCl^+$, $CdCl_3^-$)
- in the active centre of functional proteins (e.g. hemoglobin, hemocyanine), low molecular weight peptides, in the active centre of enzymes
- to low molecular weight organic acids (e.g. citrate)
- to metallothionein, to transport proteins (e.g. ferritin), or other sequestration proteins
- as intracellular granules or extracellular granules

These biochemical mechanisms serve to prevent the organism against accumulation of reactive metal species, and they might also have an impact on the accumulation level reached in organisms during exposure. In theory, when the storage compartment is inert and an infinitive sink, this will be reflected in linear uptake curves. When the storage compartment has a dynamic equilibrium with the labile compartment and elimination from the storage occurs, a saturation type accumulation curve can be seen. Adverse effects will occur whenever the metal uptake flux exceeds elimination or storage flux, and the metals

bioaccumulate in excess over the metabolically required pool or exceed a threshold concentration at the specific site of action.

In all three steps of the metal bioavailability process, metals can occur in available and non-available forms. The partitioning of metals over the different phases, solid versus soluble, is specific for each metal species and dependent on many soil and pore water properties and organism-specific characteristics (e.g. ability of uptake, uptake routes, detoxicification and excretion strategies).

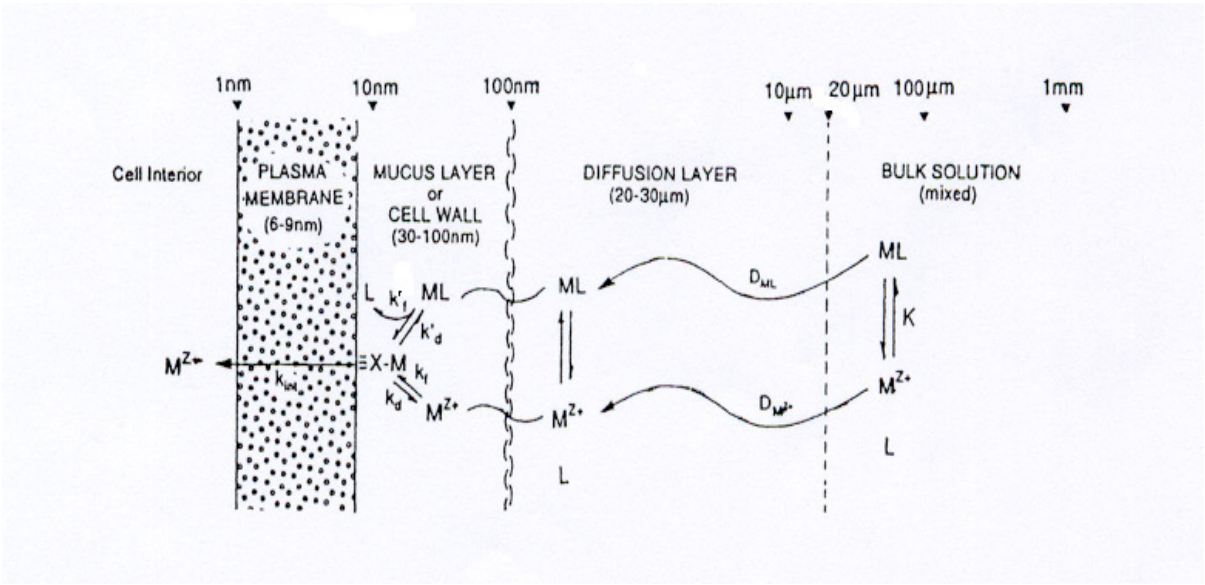

Figure 2: Conceptual model of metal-organism interaction (Campbell 1995). From the right to the left, metal M from the bulk solution enters the diffusion layer and protection layer of the animal, before transport over the membrane occurs. M^{2+} = metal cation, ML = metal bound to ligand, X = carrier ligand towards the plasma membrane, k_f and k'_f are rate constants for formation of the surface complex, k_d and k'_d are rate constants for dissociation of the surface complex. k_{int} is the rate constant for internalisation or transport of the metal across the biological membrane.

1.3 Measuring and modelling metal availability to predict bioavailability

The chemical concepts underlying metal bioavailability are extensively described by relationships dealing with the impact of soil properties on the partitioning of metals over the soil phases (Lumsdon and Evans 1995, Janssen et al. 1997, Sauvé et al. 2000, Impellitteri et al. 2002, 2003). Recent advances in analytical chemistry have markedly improved our ability to identify and measure metal species in environmental matrices. At the same time, assessment of the potential toxicity or bioaccumulation of metals by ecotoxicologists has increasingly used information on metal speciation to refine biological and biochemical toxicity models (Nolan et al. 2003). Metal uptake in aquatic systems often can be predicted using the Free Ion Activity Model (FIAM) (Morel 1983) or a related model that is based on mechanisms underlying uptake processes. In general, the interaction of metals with organisms is somehow related to a liquid phase and a (pseudo)-equilibrium is established between metal species in the bulk solution and those at the biological surface (Van Leeuwen 1999). According to the FIAM principle, metal uptake can be described as follows (see Figure 2, process depicted from right to left): First step, advection or diffusion of the metal from the

bulk solution to the biological surface. Second step, diffusion of the metal through the outer 'protective layer'. Third step, sorption/surface complexation of the metal at the carrier ligand within the protective layer, or at sites on the outer surface of the plasma membrane. And final step, uptake of the metal, transport across the plasma membrane.

In a critical review by Campbell (1995), it is shown that the FIAM theory is confirmed by many experimental data. For aquatic environments, this scientifically based theory is implemented in new regulatory guidelines for ecosystem protection in many countries (e.g. Australia, United States of America and Canada).
Unraveling metal speciation into available and non-available species in the bulk solution shows promising prospects for describing uptake in soil organisms (Sauvé 2001). However, actual implications of FIAM or other regression-based models are less straightforward compared to aquatic systems, because in soil matrices metals are present in many different chemical forms among which only a few are chemically and/or biologically available. Additional processes, such as adsorption/desorption and complexation, ensure that metal partitioning varies with time (e.g. Posthuma et al. 1998). Not only chemical availability is responsible for the difficulties in predicting metal bioavailability in soils compared to aquatic conditions, also the exposure routes of organisms to metals are more difficult to identify. Using the mechanisms behind metal interactions as described by FIAM, it is not unlikely that under low ambient (bioavailable) metal concentrations in terrestrial systems, diffusion towards the Biotic Ligand rather than metal transport over the biotic membrane is the rate-limiting step. In such environmental situations, different metal species might contribute to metal uptake. It is questionable what the impact is of metal adsorption to the organism's external membrane on uptake rates.

These uncertainties delay the implementation of metal bioavailability in risk assessment and protection guidelines. At this moment, total soil concentrations are still used to predict risks for terrestrial ecosystems and at the individual organism level. Nevertheless, science and policy explore on the bioavailability issue, by attempting to develop methods simulating available metal pools in soil. These methods, often chemical and/or model-based predictions, are aimed to be cheap, pragmatic and fast tools for quantifying environmental metal availability. Availability may for instance be determined by extracting the soil with 0.01 M $CaCl_2$ (Houba et al. 1996, Houba et al. 2000), with a 0.01 M HCl-solution (Impellitteri et al. 2003), with a diluted HNO_3-solution (Beckett 1989); pore water (total) analyses; and modelling speciation using e.g. WHAM (Tipping 1994), CHEAQS (Verweij 2000-2002), or WinHumicV (Gustafsson 1999). It is acknowledged that both modelling and measurement methods have their limitations, but model-based bioavailability predictions can be readily tested by bioaccumulation experiments. The porewater hypothesis, which assumes that metal accumulation in organisms can be predicted from metal concentrations in the water phase, can be used as starting point for this testing. The metal fraction present in the pore water is said to cause effects, irrespective whether the organism in question lives in water, soil or sediment (Van Gestel 1997). The porewater hypothesis considers all metals in solution, including

dissolved metals and metal complexed with organic and inorganic ligands. The pore water hypothesis seems to be a pragmatic tool with predictive capacity for many soil organisms (Van Gestel 1997). The theory is physiologically based and understood. Another approach showing promising results for the assessment of bioaccumulation, is the use of biomimetic techniques, such as chelex-, EDTA-resin, also called DGT (Harper et al. 1999, Davison et al. 2000, Zhang and Davidson 2000), which simulate uptake by biota at the steady state level. Nevertheless, both the physiologically-based porewater theory and the biomimetic techniques do not involve kinetics and therefore do not account for dynamic conditions.

1.4 Predicting effects from external concentrations

A method to predict acute metal toxicity to organisms from exposure concentrations is the Biotic Ligand Model (BLM), based on the idea that mortality occurs when the metal-biotic ligand complex reaches a critical concentration (Paquin et al. 2002). The chemical part of the model is formalized on the basis of the FIAM model (Morel 1983), which assumes that metal ions are the species of interest for uptake by biota. The organism is treated as a Biotic Ligand, therefore, metal uptake can be described as a competition process controlled by metal affinity with other ligands. The biological part of the BLM is based on the gill surface interaction model (GSIM) formulated for fish by Pagenkopf (1983). The gills are particularly sensitive to toxicants and the concentration in the gill is proportional to the acute effects in fish (e.g. acute effects on the respiratory system and distortion of osmotic balance). BLM model predictions are accurately working for fish, and also for organisms without a gill, on the condition that the organism is in direct contact with the external aqueous environment (Di Toro et al. 2001, Heijerick et al. 2002, De Schamphelaere and Janssen 2002). Although in these studies the similarity of conditional binding constants (K of metal-BL) for a range of organisms indicates that the mechanism of binding is similar over many biota, it is also recognized that the effect of water chemistry on metal toxicity is dependent on the organism studied. Terrestrial BLM, like the FIAM approach, deal again with more difficulties than their aquatic counterparts due to the soil matrix, and the BLM formulations are up-to-now regression-based descriptions not having the toxicological foundations as for fish (Pagenkopf 1983). The first building blocks for a terrestrial BLM were obtained for the springtail *Folsomia candida* (Van Gestel and Koolhaas 2004), and here pH and, to a minor extent, Ca were predictive for the toxicity of Cd.

1.5 Predicting effects from internal concentrations

A general accepted approach for assessing possible adverse effects on biota, no matter what kind of species, is the Critical Body Residue (CBR) concept (McCarty 1991). The CBR is defined as the highest internal concentration of a substance in an organism that does not yet cause an adverse effect. By comparing measured internal concentrations to CBR values derived in the laboratory, a measure of risk is obtained. An advantage is that the CBR concept integrates environmentally available fractions with bioavailable concentrations, and toxicity at specific receptors (McCarty and MacKay 1993). In principle, the actual exposure concentration in the environment does not need to be known to allow for a risk assessment, and therefore many difficulties are overcome regarding bioavailability issues, e.g. it removes

some of the disadvantages of the exposure concentration expressed per unit of soil (Van Wensem et al. 1994), as well as variable exposures (Hickie et al. 1995). A convincing body of evidence was collected to support the CBR approach. Especially for organic compounds, effects could be assessed over a wide range of organisms, compounds tested and exposure media (e.g. Lanno et al. 1998, Parkerton and Konkel 2000, Fay et al. 2000). However, also critical remarks have been made. Crommentuijn et al. (1994) concluded that critical body burdens of metals in soil invertebrates are species-specific. Adaptation processes limit the application of CBRs for metals (Indeherberg et al. 1999), and it was found that the way organisms deal with accumulated metals has a large impact on the magnitude of body concentrations reached and the accompanying metal sensitivity (Rainbow 2002). When the internal metal concentration does not show a monotonic relationship with the exposure concentration, it is not possible to derive CBRs. This means that whenever organisms are capable of trapping a portion of the metal in forms that are not biologically reactive, a direct relationship between metal tissue concentrations and toxic effects may not exist or may be less evident. Consequently, a wide range of body concentrations with different biological significance exists.

1.6 Animal physiology in relation to bioaccumulation and effect

For setting soil quality criteria, it is desirable that a variety of organisms are studied, that jointly give an indication of ecological responses to the contaminant (Van Straalen and Van Gestel 1993). One of the criteria is acknowledging the existence of organisms for which uptake via the pore water is obvious and organisms for which uptake via other phases, e.g. explicit solid phases, can be of importance. Hence, especially in terrestrial organisms, it is likely that routes other than pore water are contributing to metal uptake. After all, to protect themselves from desiccation, soil-dwelling organisms inhabiting the top soil layers often have a less water-permeable epidermis.

The earthworm species *Lumbricus rubellus* and *Aporrectodea caliginosa*, on which this thesis focuses, represent animals that are in close contact with the pore water of soil and have a water-permeable surface epithelium. The isopod *Porcellio scaber* represents an animal having less obvious contact with the soil pore water and is protected by a calcium-rich integument.
Oligochaete worms have a thick mucus layer that surrounds the epidermis (Laverack 1963), through which respiration and the excretion of waste products occur. This mechanism makes the earthworms sensitive to water loss. The digestive interior of oligochaete species is well investigated (Wallwork 1983). There is evidence that the uptake of food via the gut is not a heterogeneous process during the gut passage. During ingestion mucus is mixed with the food. In the first part of the digestive system of an oligochaete, calciferous glands actively release Ca^{2+} in the gut contents. The crop is used for storage of the gut content, before mechanical grinding and digestion in the gizzard. The gizzard opens up into the intestine, which forms the largest part of the alimentary canal. Gut conditions in the final part of the digestive system (the intestine) are actively regulated by excretion of NH_4^+. A typhlosole (see Figure 3), a dorsal infolding of the gut epithelium effectively increasing the internal surface,

is present along the anterior and mid intestine, thereby also increasing the secretory and absorptive surface areas. The pH along the entire digestive tract is quite constant between 6 and 7, and the digestion is driven by enzymes (Edwards and Lofty 1972). The gut pH is often higher than the bulk soil pH, especially in earthworms inhabiting acid soils.
Earthworms are able to accumulate metals to a great extent. The ability to deal with high levels of accumulated metals can be ascribed to the slow turnover of the tissues in which metals accumulate. Metals, such as Cd and Cu, are predominantly bound to metal-binding proteins (Stürzenbaum et al. 2001) and with these proteins, the metal moves through the body to organs and tissues in which it is deposited in inorganic forms. Cd was retrieved in high amounts from the nephridia and to a lower extent from the body wall of earthworms (Prinsloo et al. 1999). Pb is found in waste nodules located in the coelomic fluid (Andersen et al. 1982).

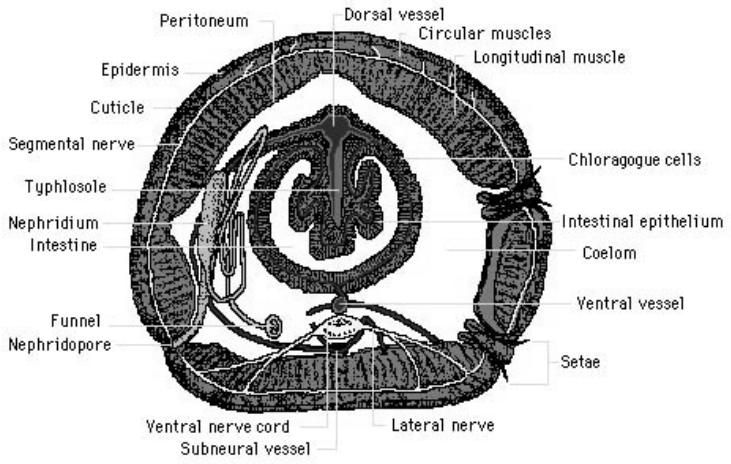

Figure 3: Cross section at height of the posterior intestine of earthworms (www.biodidac.bio.uottawa.ca)

The largest part of the body burden is bound in the chloragogenous tissue (Ireland and Richards 1981) located around the digestive tract (see Figure 3). The cells of this tissue (chloragocytes) contain many chloragosomes, including calcium granula (type A) and sulphur-rich granules (type B). All granulum types are likely to play a role in the homeostasis of essential metals. Nevertheless, nonessential metals do enter the body and will be partitioned over the tissues and organs based on their relative affinity for biomolecules. For instance Cd preferentially binds to sulphur-rich granules instead of oxygen-rich granules, and hence is found in the type B granules, also called cadmosomes. Especially in the posterior alimentary canal, elevated concentrations of Cd, Pb, Zn and Ca can be found, located in the chloragogenous tissue (Morgan and Morgan 1990, 1998). This might be explained by the resorption capacity of the digestive tract that is most efficient in the posterior alimentary canal.

Terrestrial isopods have their evolutionary origin in the marine environment and this is still recognizable in some aspects of their physiology. Most isopods are very sensitive to desiccation and an efficient water regulation within the excretory system is essential. Moreover to prevent too much transpiration, isopods have organs, such as gills, still functioning as the respiratory system (Sutton, 1972), which are constructed to allow gas

exchange almost without water loss. The digestive system of the isopod is extensively described (Hames and Hopkin 1991) and is divided into the foregut, the anterior chamber, the papillate region, the rectum of the hindgut and the hepatopancreas. Various parts of the gut show different enzymatic activities, thereby having slight changes in pH. Nevertheless, the isopod does not have acid digestion for nutrient uptake (Warburg 1993). Food passes via the oesophagus to the foregut, where it is mixed with enzymes from the hepatopancreas, before passing into the anterior chamber of the hindgut. Contraction of the muscles surrounding the gut forces liquids and food particles back into the foregut via the typhlosole channels. There they are filtered and passed on to the lumen of the hepatopancreas where nutrient absorption takes place.

Isopods accumulate metals to a high extent, and are able to deal with high body concentrations due to a detoxification strategy based on accumulative immobilization. The efficient storage organ is the hepatopancreas covered with certain S (small) and B (big) cells (see Figure 4), which contains many granules (Hopkin et al. 1989, Hopkin 1989).

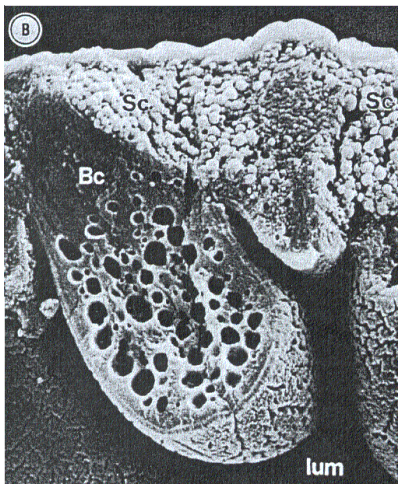

Figure 4: Scanning electron micrograph of a B cell (Bc) and an S cell (Sc) in the hepatopancreas tubule of an isopod (taken from Hopkin et al. 1989).

Metals are sequestered, based on their binding preference, by the calcium-rich (type A), sulphur-rich (type B) and ferritine-rich granules (type C). These storage granules keep the metal concentration in the body fluid low. The S and B cells of the hepatopancreas have a dominant role in the metal turnover kinetics. B cells show a daily cycle of ejecting their contents into the lumen of the hepatopancreas, while the shape and content of S cells remains the same (Hames and Hopkin 1991). This suggests that B cells have an impact on metal excretion, while the S cells influence the metal storage.

1.7 Aims of the thesis

Summarizing the available literature on metal accumulation and risks, it is concluded that uncertainties still exist on predicting adverse effects of metals for biota when exposed to metal-polluted soils, especially under changing environmental conditions. Information is lacking on the impact of metal speciation on uptake kinetics, and on the toxico-dynamic behaviour of metals inside the organism’s body. In this thesis, some of the issues of

bioavailability of metals to soil invertebrates are addressed. The specific goals of this thesis were:

1. to link metal speciation to metal uptake by soil invertebrates
2. to link metal uptake to the ecophysiology of soil invertebrates, in particular of internal compartmentalization of metals
3. to derive a transfer function for metals from soil to soil invertebrates.

As indicated above, the species used in this thesis to investigate metal uptake and elimination kinetics and detoxification strategies, were selected from a physiological point of view. Different earthworm species (*Lumbricus rubellus* and *Aporrectodea caliginosa*) were chosen because of their differences in pedo-ecological classification (according to Bouché 1977) including habitat and feeding behaviour. Like the epigeic earthworm, the isopod *Porcellio scaber* is a topsoil layer and litter-dweller. The physiological differences and the difference in prefered habitat may result in different possibilities of metal uptake from the soil.

- *Lumbricus rubellus* (Hoffmeister) (Figure 5) is an epigeic earthworm species, which inhabits the top soil layers and litter layers. The animal feeds mainly on litter and organic-rich material. They are typically small in size (length 3-5 cm) and pigmented. This group does not enter summer diapause under harsh conditions (Sims and Gerard 1985).

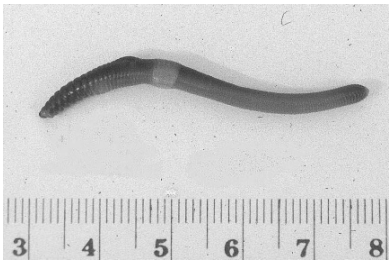

Figure 5: Earthworm *Lumbricus rubellus*

- *Aporrectodea caliginosa* (Savigny) (Figure 6) is an endogeic earthworm species, which characteristically constructs burrows in highly mineralised soil horizons and mainly feeds on organic-rich soil. They are weakly pigmented and vary from size (2-8 cm). This group can enter obligatory or facultative diapause under adverse environmental conditions, such as drought or flooding (Sims and Gerard 1985).

Figure 6: Earthworm *Aporrectodea caliginosa*

- *Porcellio scaber* (Latreille) (Figure 7) is a crustacean species that commonly exists in the top-soil and litter layers. The animal prefers moist environments. The grey color of the thorax

and the shape of the uropods can characterize this species. The actual size of the isopod is about 15 mm, with female isopods being larger than male.

Figure 7: Isopod *Porcellio scaber*

Both earthworms and isopods inhabit the flood plain ecosystem and are in close contact with soil. Earthworms play a prominent role in the soils, due to their importance for soil fertility as being soil engineers. By digging burrows, the soil is aerated and different soil layers are mixed. The feeding behaviour of earthworms is selective, litter and leaves are fragmented and mixed in with the soil (Edwards and Lofty 1972). Earthworm bioturbation makes the soil accessible to microbes and fungi, and thereby creates a healthy ecosystem. The abundance and relative biomass makes earthworms an important functional group, and adverse effects on these organisms will have an impact on the entire ecosystem. Isopods are important especially for fragmentation of litter material and in this way contribute to decomposition. Both species play a key role in natural food chains providing a food source for many small mammals and birds. The wide distribution of the test organisms chosen and their ecotoxicological relevance makes them suitable as bioindicator species in the field (Løkke and Van Gestel 1998).

1.8 Outline of the thesis

To understand how metals are taken up, accumulated and to which extent, the following stepwise approach was used in this thesis:

In **Chapter 2**, results of monitoring different floodplain systems during three year-cycles are reported. The biological variation in internal metal concentrations in two physiologically different earthworm species and various isopod species was characterized. Attempts were made to identify the possible causes of these internal variations over time in the different floodplain soils.

To understand the underlying mechanisms of metal uptake by biota, laboratory and literature studies were performed.

In **Chapter 3** the importance of dermal and oral uptake was investigated for the earthworm *Lumbricus rubellus* by blocking the earthworm's mouth. It was concluded that pore water uptake, via ingestion and oral uptake of solid particles, contributed little to metal accumulation. The dermal route was of most importance for metal bioaccumulation in earthworms.

In **Chapter 4** uptake and elimination rate constants for metal accumulation in the earthworm *Lumbricus rubellus* were derived. Metal bioaccumulation in the earthworms appeared to

require at least two-compartment modelling to account for the physiological aspects of internal metal distribution over biologically active and inert fractions. Moreover, the total bioaccumulation of metals in the earthworms could not be explained solely from the soluble metal pool in the soil.

In **Chapter 5** uptake and elimination rate constants for metal accumulation in the isopod *Porcellio scaber* exposed either to contaminated soil or contaminated food were derived. Metal accumulation in animals exposed to a combination of food and soil was estimated using the kinetics derived from the single exposure experiments and was shown to be additive. Uptake rate constants from different sources (food versus soil) were shown to be equal. The magnitude of steady state levels reached was driven by the concentration in the exposure medium and the differences in elimination kinetics influenced by metal distribution in the isopod's body.

In **Chapter 6** surface adsorption of metals onto the earthworm *Lumbricus rubellus* and the isopod *Porcellio scaber* was distinguished from metal absorption in the body, and was shown to be negligible. In this way, more insight was gained in the uptake rate-limiting step and Cd and Zn localization in organism's bodies was determined.

Chapter 7 gives an overview of the internal compartmentation strategies of metals, based on biochemical binding capacity and metal affinity of certain organs and tissues, in invertebrate species. The relevance of internal compartmentation for ecological risk assessment was discussed.

In **Chapter 8** the internal metal compartmentalization was qualified and quantified in the earthworm *Aporrectodea caliginosa* exposed to different field soils. Metals bound to proteins could best explain the accumulation pattern over time. The granular fraction showed to have the lowest elimination capacity of all internal fractions.

Chapter 9 provides a general discussion of this thesis. An overview is given of the main findings in the field monitoring and the scientific knowledge obtained from the laboratory experiments. This synthesis resulted in two transfer functions in which bioaccumulation in soil-dwelling invertebrates is linked to metal availability in the soil. The two transfer functions take into account differences in routes of uptake. The mechanisms of bioaccumulation are generalized and made applicable for risk assessment. The resulting tool may assist decision makers, landowners and researchers as a decision support system for assessing effects of metal contamination in terrestrial ecosystems.

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Chapter 2

Monitoring metal accumulation in earthworms and isopods inhabiting floodplain soils

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Chapter 2
Monitoring metal accumulation in earthworms and isopods inhabiting floodplain soils

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Abstract

To investigate the main factors contributing to variation in metal concentrations in earthworms and isopods inhabiting floodplain soils, a three-year monitoring programme was performed. Three different floodplains were selected located along the rivers Nieuwe Merwede and Waal (The Netherlands), differing in inundation frequency and vegetation type. At each sampling time, As, Cd, Cr, Cu, Pb, Zn, Fe and Ca concentrations in earthworms and isopods were determined. Various external factors were analysed, including metal concentrations in soil and pore water, soil properties, litter availability, flooding frequency, and river water quality. Metal concentrations in the epigeic earthworm *Lumbricus rubellus* showed large seasonal variations, whereas the endogeic earthworms *Allolobophora chlorotica* and *Aporrectodea caliginosa* showed less seasonal variation. Differences in internal levels between sampling intervals were largest in earthworms from the floodplain sites frequently inundated. High and low frequency flooding did not result in structural changes in internal metal concentrations. A presence of litter did not affect metal levels in *L. rubellus*, except for Cd that increased. Total concentrations in soil as a single descriptor did not explain metal concentrations in epigeic and endogeic earthworms, whereas soluble metal pools in the soil could explain some of the variation in internal levels. Internal level of most essential metals coincided with earthworm activity. Metal concentrations in isopods neither differed between the species nor between the sites. Seasonal variation in isopods was found only for Pb and in some cases for Ca, Cd and Zn, although an external factor explaining these variations could not be identified. Metal bioaccumulation is believed to be dependent on different factors that interact with each other in a very complex manner. Metal concentrations in the earthworms and isopods can be seen as an integration of fluctuations of all external factors.

2.1 Introduction

Impact of human activities on river systems has been noticeable for at least the last 200 years. Although local contamination with metals already started in about 1900, pollution degree increased around the 1970s, due to extensive industrialisation, mining activities, wastewater discharge and agricultural activities. Contaminants, such as metals, adsorb to suspended particles, which settle under low stream velocities. This retention of contaminants is almost permanent when sediments are deposited in the floodplains, increasing soil concentrations to high levels, often above tolerable quality standards. As a result of international efforts, concentrations of metals have decreased considerably until the late 1980s (De Boo and Middelkoop 1999), and since that time the polluted floodplain soils are covered with cleaner sediment. Pollution history is reflected in the pollution levels at different depths in the sediment (Beurskens et al. 1993).

It is relatively unknown how raised metal levels affect the organisms inhabiting the flood plain systems. Despite high contamination levels, the properties of floodplain soils, such as high pH and high organic matter content, cause low soluble metal concentrations (Janssen et al. 1997, Sauvé et al. 2000, Van Leeuwen 1999), which is believed to be an important source of exposure for most organisms (Van Gestel et al. 1997). However, in earthworms inhabiting Dutch floodplain areas elevated metal concentrations were detected, whereas in isopods and millipedes having a less water-permeable epidermis, metal levels were similar to species found in unpolluted reference areas (Hobbelen et al. 2004). The explanation of this controversy remained indistinct, probably because of the involvement of many interfering factors.

In the literature, field studies in which internal and external metal concentrations are measured, usually have a static character, whereas the floodplain ecosystems by definition are subjected to fluctuating conditions. We tested the hypothesis whether inundation influencing the availability of metals also is reflected in metal accumulation by biota. Moreover, the metal accumulation was related to the habitat of the organisms. To investigate the temporal dynamics of metal bioaccumulation, the epigeic *Lumbricus rubellus*, the endogeic *Allolobophora chlorotica* and *Aporrectodea caliginosa* and several isopod species were monitored with a two to five months interval over a period of three years. Both species are key species in invertebrate and vertebrate food chains. The epigeic species have their habitat in the top layers of the soil and in litter layers on the soil, whereas the habitat of endogeic species is more in the deeper soil layers (Bouché 1977). Isopods inhabit the top soil layers and the litter on the soil. Three different floodplain sites were selected characterised by differences in inundation frequency and vegetation types. The internal metal concentrations in the animals over time were related to external factors, such as soil properties, metal concentrations, river water quality, seasonal changes in vegetation, and inundation.

2.2 Materials and methods

Research sites and abiotic parameters

Three sites along side the rivers Nieuwe Merwede and Waal were selected based on soil characteristics and vegetation type. Figure 1 gives a map of the major Dutch rivers, the floodplain sites monitored are marked. River Waal switches over to Nieuwe Merwede, and therefore has the same origin. Geographical information on the sites and vegetation types are summarized in Table 1.

Table 1: Site characteristics. X and y coordinates were determined using a GPS-system calibrated at Amersfoort, The Netherlands. NAP = Normaal Amsterdams Peil (Dutch reference mean sea level). Mixed vegetation includes reed *Phragmites australis*, herbs e.g. *Urtica dioica*, *Symphytum officinale* and *Valeriana officinalis* and shrub species e.g. *Salix*. The grassland vegetation mainly consists of grass and some *Phragmites australis*.

Site code	Location	x-coordinates	y-coordinates	Height above NAP (cm)	Rivers	Vegetation type
M	Lage Hof	111.314	420.428	85	Nieuwe Merwede	Grassland
P	Ruitersplaat	111.235	420.000	105	Nieuwe Merwede	Mixed vegetation
S	Stiftse Waard	155.240	427.360	457	Waal	Grassland

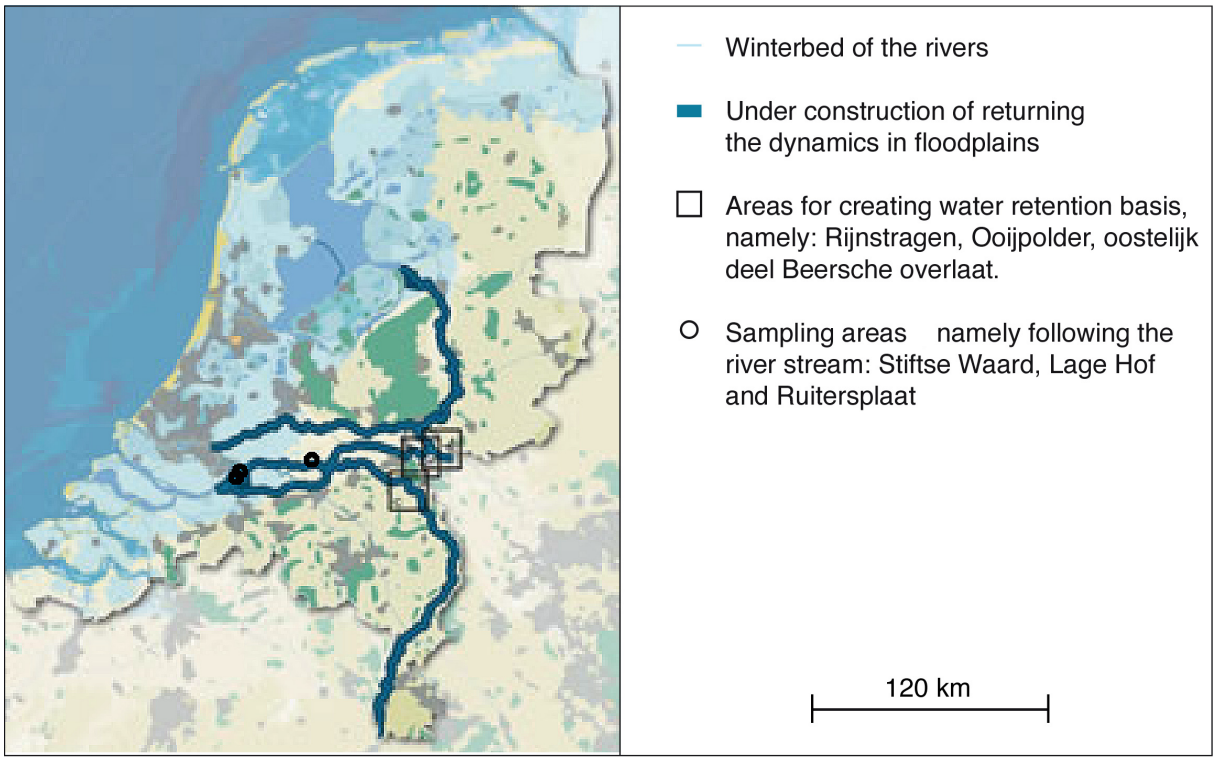

Figure 1: An overview map of the major Dutch rivers for which the Ministry of Transport, Public works and Water Management (Rijkswaterstaat) is responsible. Sampling sites are marked with an open circle.

Cattle grazed the sites M, P and S. The sites were located on gradually sloping riverbanks, and were subjected to periodic inundation. The plots from which the earthworms and isopods were sampled, measured 10 by 10 meter. Fluctuations in water level and inundation frequency are depicted in Figure 2. Inundation patterns in the fresh water estuary floodplain M and P are influenced by sea tides and wind, an example of a typical cycle is given in Figure 2 (inset). Water levels were monitored during 2000-2004 by Rijkswaterstaat and are available on www.waterbase.nl. For the sites M and P the nearest monitoring station is “Werkendam buiten” and for the site S the nearest monitoring station is “Zaltbommel”.

At all sites we measured inundation frequency, water quality of the rivers, metal concentrations in soil and pore water, soil and pore water properties, litter and organisms. Quality of the river water (Waal and Nieuwe Merwede) flooding over the sites was already monitored for several years by Rijkswaterstaat before the start of our sampling period, 1998-2001. Soil characteristics and total metal concentrations were determined in November 1999 and August 2000. Pore water was collected using different techniques. At all three sites, pore water was collected by centrifugation. The pore water samples were filtered over 0.45 µm before chemical analysis. In addition, at site M pore water was monitored *in situ* regularly from November 2000 to December 2001, using a permeable pore water sampler (Rhizon SMS-MOM, Rhizosphere Research Products, Wageningen, The Netherlands). At site P, the sampling was done under anaerobic conditions.
Organic litter was collected three times in 2003 and 2004 for metal analyses.

Collection of organisms
From November 2000 to February 2004, earthworms and isopods were collected from the floodplain sites M and P at intervals of three to five months. At site S, earthworms were collected in 2000-2002 only. At all sites collection was random within the 10×10 meter plot. After taking the organisms to the laboratory, earthworms were allowed to defaecate for 48 hours and isopods for 28 hours. Their wet weight was determined, followed by storage in a freezer at –18 °C until analyses.

Chemical analyses
Animals and litter were freeze-dried and dry weights were determined. The lyophilized organisms were digested in a concentrated HNO_3 solution using a Mars5 destruction microwave oven and measured by ICP-MS (Perkin Elmer, SciEx ELAN 6000, Concord, Ontario, Canada). Soil samples were dried at 40°C and sieved (< 2 mm) before analysis. As, Cd, Cr, Cu, Pb, Zn, Mn, Fe concentrations in *aqua regia* (NEN 6465, 1992) were measured by ICP-AES (Spectros, Specto Flame) and ICP-MS. Certified reference material Dolt-2 (BCR, Brussels, Belgium), and blancs were treated similar as the samples. No systematic correction was applied to the body residue analyses, since recovery of the standard addition was within acceptance limits (90-110%). Measured metal concentrations of the standard reference were within performance acceptance limits (95-105%). The organic carbon content of the soils was analysed by wet oxidation with $K_2Cr_2O_7$ (Walinga et al. 1992). Clay content was measured by sedimentation according to Houba et al. (1996).

Statistics
Internal metal concentrations of organisms were tested for outliers using the Grubbs test (Funk et al. 1985). For each group of animal species, homogeneity of data was tested using Fmax, followed by analysis of covariance (ANCOVA) to correct metal accumulation for body weight (dependent = internal concentration and covariable = weight and factor = time). For each group of animal species, one-way ANOVA tested the significance of time of measurement (dependent = internal concentration and factor = time) followed by the Tukey

Post-Hoc test to analyse for significance of the differences between the seasons ($p<0.05$). All statistics were performed using the software packages Systat 9.0 and SPSS 10.0.

2.3 Results and discussion

Sites M, P and S differed in inundation frequency (Figure 2). Due to the influence of sea tides, M and P were flooded periodically during the whole year, but were only permanently inundated during some winter periods. Flooding of site S was long-lasting and occurred usually in winter and early spring, and lasted for a period up to three months depending on rainfall.

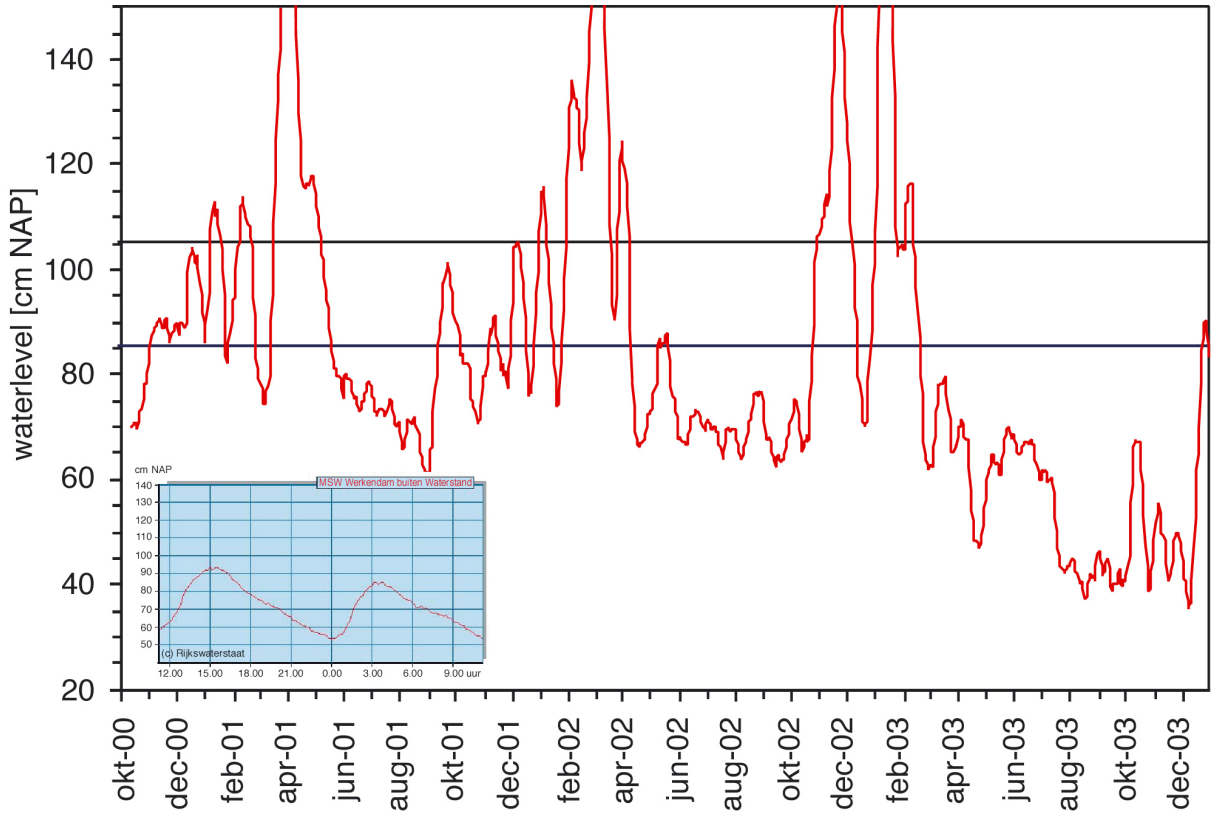

Figure 2: Average water level measured at Werkendam buiten, The Netherlands. The water level is relative to the Dutch reference sea level (NAP) and averaged over a 14 day interval. The horizontal lines represent the height of the sampling sites M (at 85 cm) and P (at 105 cm). These floodplains are also subjected to a daily flooding cycle that is shown in the inlet. Source: Dutch Ministry of Transport, Public Works and Water Management (RWS).

Water quality did not differ between the rivers Waal and Nieuwe Merwede, and data are therefore not shown.

Soil characteristics and total metal concentrations are summarized in Table 2. Pollution levels of the three floodplain soils are specified as pollution class 4, which means that the metal concentrations exceed the intervention values according to the Dutch regulations (4th National Policy Document). To compare metal levels with these trigger values (target and intervention), a correction for clay and organic matter content was applied as described by Vegter (1995).

Table 2: Soil properties and total metal concentrations at three sites expressed on a dry weight basis. The metal concentrations exceeding the Dutch target values for soil are *italic*, the concentrations exceeding the intervention values are ***bold***. Calculation of exceeding trigger values was done by correction for clay and OM content as described by Vegter (1995).

site	OM	<2µm	pH	As	Cd	Cr	Cu	Pb	Zn	Fe	Ca
	[%]	[%]	0.01M $CaCl_2$	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[g/kg]	[g/kg]
M	10.5	32.5	7.6	144	***16.8***	*507*	***316***	***561***	***2308***	41.1	42.9
P	19.0	34.9	7.2	195	***18.5***	***527***	***343***	*551*	***2494***	n.d.	n.d.
S	2.1	19	7.5	24.2	*1.79*	75.0	*43.9*	*116*	*353.6*	20.2	43.7

Site P is the most polluted location followed by site M and site S. Concentrations of Zn, Cd, Cu, Cr and Pb exceed the intervention values in most cases at sites M and P, and target values at site S. This suggests that biota can be affected at all sites.
A summary of the metal concentrations measured in pore water at the different sites is given in Table 3.

Table 3: Metal concentrations in pore water sampled in floodplain sites M, P and S. The concentrations exceeding reference values in Dutch soil protection policy are **bold**.

site	sampling	time	DOC	pH	Ca	Fe	Cu	Zn	Cd	Pb	As	Cr
	method	(month-yr)	[mg/l]	0.01 M $CaCl_2$	[mg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]
M	rhizon	Nov-01	24.4	7.52*	164	582	28.3	482	3	2.6	18.6	3.2
M		Feb-01	13.7	7.03	142	767	22.6	720	**5.67**	1.94	15.7	4.2
M		May-01	12.5	7.34	160	1144	22.3	580	**5.69**	1.26	17.5	2.3
M		Sep-01	16.5	6.87	187	d.l.	53.6	759	**7.48**	1.39	17.1	6.1
M		Dec-01	11.2	7.07	172	382	27.4	727	**5.52**	2.11	15	5.8
M	anaerobic	Aug-00	28.8	7.88	135	d.l.	57.1	168	2.92	1.56	12.6	n.d.
P		Aug-00	23.5	7.52	111	d.l.	57.1	225	2.74	1.87	8.82	n.d.
M	centrifugation	Feb-01	23.3	7.51	109	<500	42.2	308	3.71	1.28	9.81	n.d.
S		Feb-01	20.5	7.69	88	<500	13.6	144	0.6	0.62	4.28	n.d.

n.d. = not determined, d.l. = below detection limit, * measured in laboratory instead of in situ.

Only Cd concentrations in the pore water of site M were elevated compared to soils used as control in laboratory experiments or sampled in reference sites (compared to values summarized by Hobbelen et al. 2004). Pore water concentrations of all other metals ranged within the boundaries of reference values. High organic matter content and high pH, characteristic for floodplain soils, suggest a strong sorption of metals (Wolt, 1994) and can partly explain the low pore water concentrations.
Litter layer was collected from sites M and P, in May 2003, November 2003 and February 2004. The metal concentrations are given in Table 4.

Table 4: Metal concentrations (dry weight ± stdev, n=3) in the litter layer of the floodplain sites M and P.

site	time	As	Cd	Cr	Cu	Pb	Zn	Fe	Ca
	(month-yr)	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
M	05-2003	27.3 ± 0.98	4.79 ± 0.16	153 ± 5.63	110 ± 3.50	124 ± 3.87	818 ± 34.2	26445 ± 991	33827 ±
	11-2003	38.6 ± 0.93	5.66 ± 0.19	162 ± 6.07	115 ± 2.68	143 ± 4.36	895 ± 8.21	22045 ± 829	43635 ± 2834
	02-2004	69.2 ± 7.54	9.00 ± 0.73	290 ± 29.8	189 ± 20.2	239 ± 27.3	1334 ± 129	31624 ± 3222	46590 ± 3556
P	05-2003	37.2 ± 4.63	5.74 ± 0.64	118 ± 12.8	85.6 ± 8.01	156 ± 18.3	1226 ± 133	21577 ± 2313	59076 ± 2978
	11-2003	43.8 ± 17.4	10.5 ± 3.70	169 ± 60.7	120 ± 41.2	185 ± 68.2	1617 ± 599	14837 ± 5144	61572 ± 21599
	02-2004	49.4 ± 7.18	7.70 ± 1.25	156 ± 24.9	120 ± 19.6	193 ± 30.1	1491 ± 225	19810 ± 3110	38149 ± 6995

Earthworm species were grouped in epigeic and endogeic species on the basis of their burrowing and feeding behaviour (Bouché 1977). Metal concentrations in the epigeic

Lumbricus rubellus collected from sites M and P, from November 2000 to February 2004, and from site S from November 2000 to December 2001, are shown in Figure 3. Figure 4 shows the metal levels in earthworm species *Allolobophora chlorotica* and *Aporrectodea caliginosa* sampled from site M, P and S from November 2000 to May 2001. No difference in internal Ca, Cu, Fe, Zn, Pb, Cd, As and Cr concentrations (ANOVA, $p > 0.05$ n=35) between the two species was observed. Within the statistical analyses to test on variation of internal metal levels over the seasons and identifying the external parameter causing the variation, the two endogeic earthworm species were grouped. Metal concentrations in isopod species collected from November 2000 to February 2004 are shown in Figure 5. *Trachelipus rathkii, Philoscia muscorum, Oniscus asellus* and a group of not identified isopod species were sampled from sites M and P. All species were treated as one group, since no variation between species was observed.

Calcium

In general, variation of Ca concentrations between *L. rubellus* individuals was small. Compared to maximum Ca concentrations of 8542 µg/g found in earthworms from non-polluted areas (Van Gestel et al. 1992), the concentrations were only slightly elevated. Significant variations in internal Ca concentrations exist over the seasons (ANOVA, $p < 0.05$). Earthworms sampled from site M showed significantly higher concentrations in March 2003 (Tukey, $p < 0.05$) compared to the autumn and winter months. Earthworms sampled in November 2000 and May 2001 showed significantly lower concentrations. In earthworms collected from site P in May 2003 significantly higher internal concentrations were found compared to the autumn and winter months (Tukey, $p < 0.05$). At sampling site S earthworms showed significantly lower concentrations in November 2000 and February 2001. In general, a uniform pattern for Ca for all sites was found. None of the environmental factors, such as inundation frequency, availability of litter layers, total and pore water concentration, pH, soil and pore water characteristics, could explain the seasonal variations in internal Ca concentrations. It might be that earthworm activity (burrowing and reproduction), which is in general shown to be lower in the colder months, explains part of the variation. However, variation cannot solely be attributed to seasonal activity differences, see for example internal levels determined in earthworms collected in May 2001, site M. For the endogeic earthworm species also significant variations in internal Ca concentrations existed over the seasons (ANOVA, $p < 0.05$). However, none of the seasons could be identified having different internal Ca levels (Tukey test, $p > 0.05$). Compared to the epigeic earthworm having Ca levels ranging from 6 to 18 mg/g d.w., Ca concentrations in endogeic earthworm species exposed in the three floodplain sites were lower, ranging from 2 to 10 mg/g d.w. The difference between the species may be ascribed to highly active calciferous glands of *L. rubellus* and inactive calciferous glands of *A. caliginosa* and *A. chlorotica* (Piearce 1972). It can be concluded that Ca levels in the earthworms *A. caliginosa* and *A. chlorotica* are elevated compared to *A. caliginosa* found at unpolluted sites, because these internal levels range from 1.7 to 2.7 mg/g d.w. (Morgan and Morgan 1998).

Ca concentrations in isopods differed significantly between sampling times (ANOVA, $p < 0.05$). For animals from site M, however, no significant differences could be found over the seasons (Tukey, $p > 0.05$). Isopods collected from site P showed significantly higher Ca concentrations in May 2003 and November 2003 (Tukey, $p < 0.05$). Isopod concentrations were significantly higher in September 2001 than November 2000 (Tukey, $p < 0.05$).

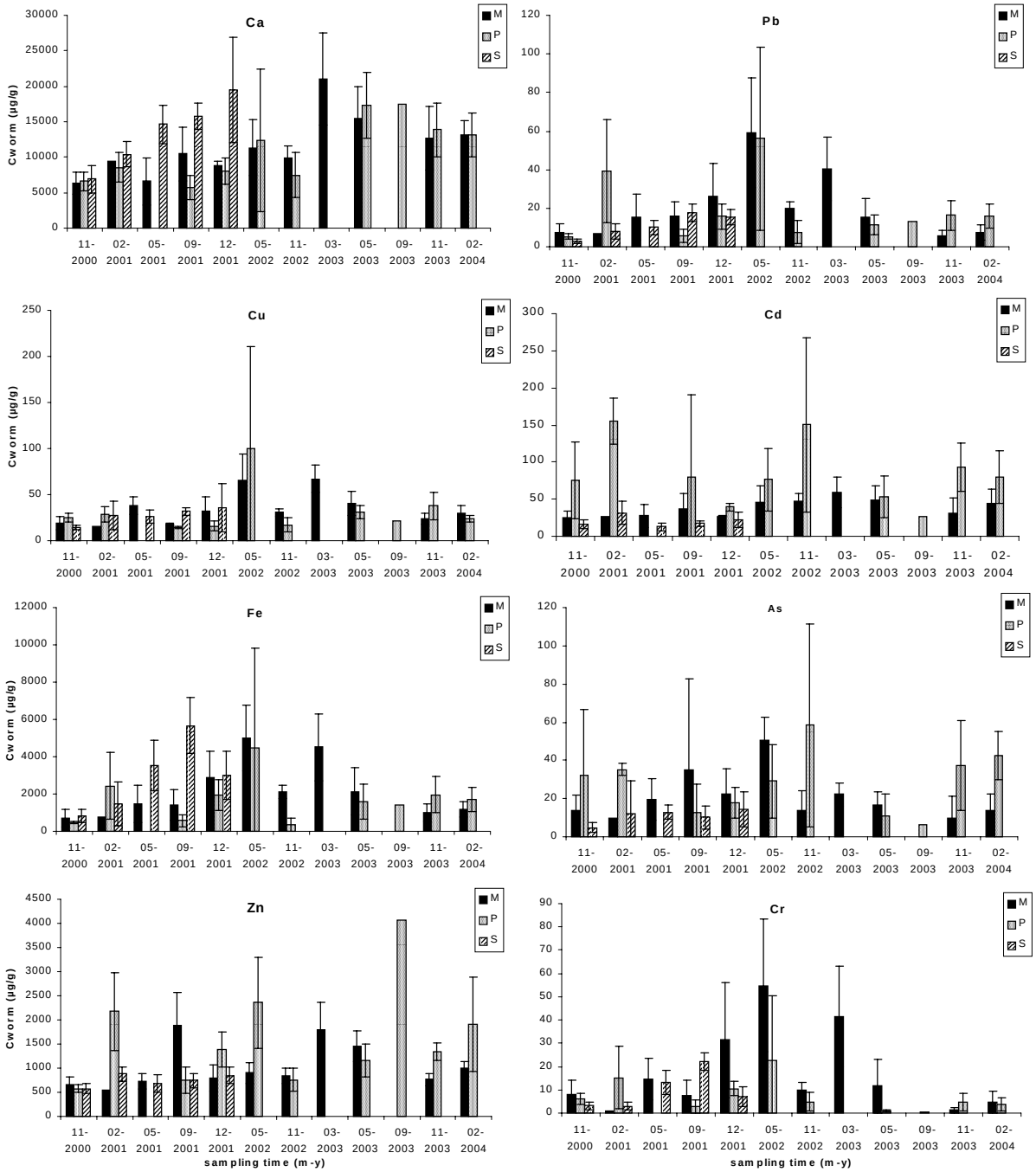

Figure 3: Metal concentrations (on dry weight basis ± stdev) in *Lumbricus rubellus* sampled from different floodplain sites over time (month-year). In the left column internal concentrations for the essential metals Ca, Cu, Fe and Zn are shown, in the right column the non-essential metals Pb, Cd, As, Cr.

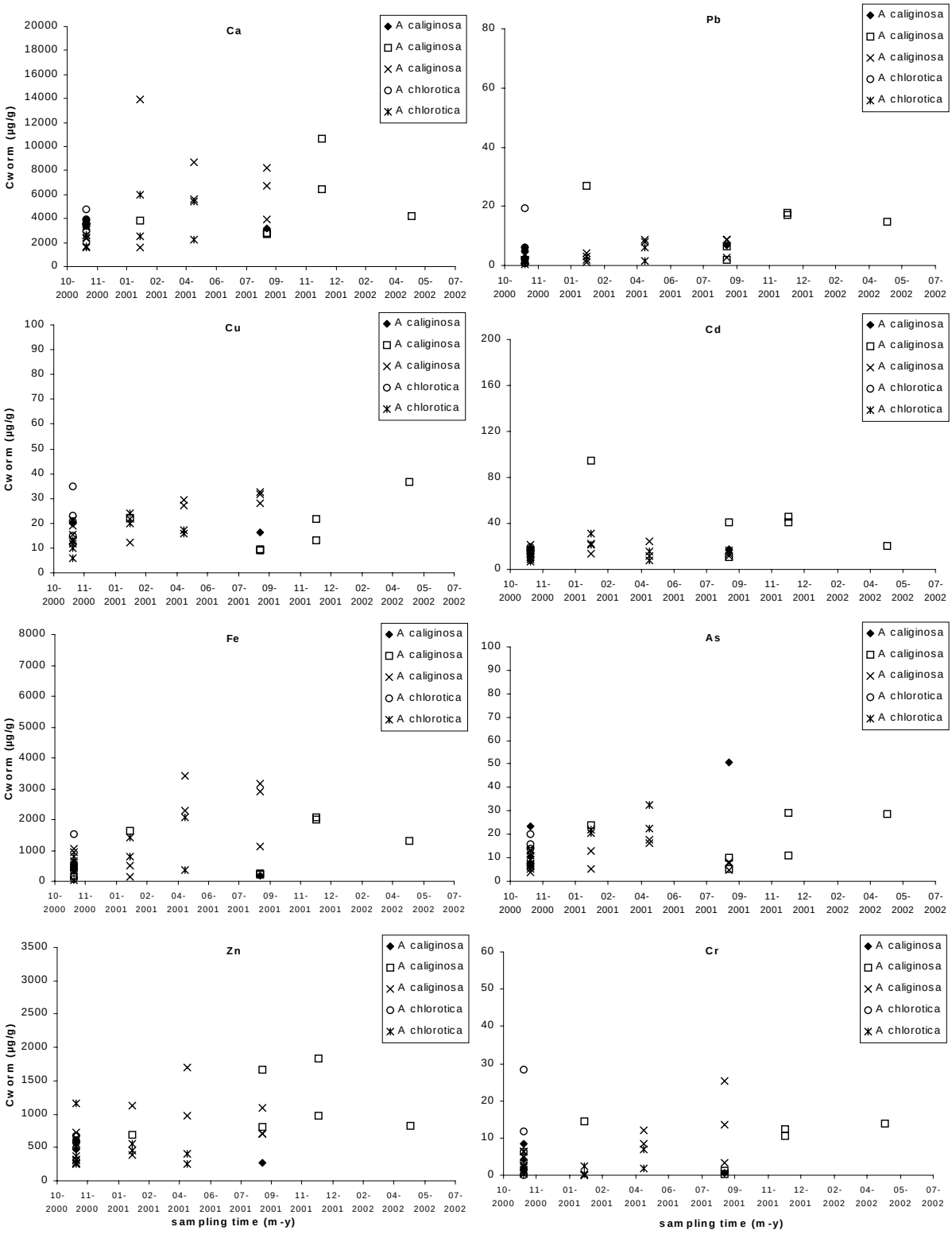

Figure 4: Metal concentrations (on dry weight basis ± stdev) in *Allolobophora chlorotica* and *Aporrectodea caliginosa* sampled from different floodplain sites over time (month-year). In the left column internal concentrations of the essential metals Ca, Cu, Fe and Zn are shown, in the right column the internal concentrations of the non-essential metals Pb, Cd, As, Cr. The solid symbols represent earthworms collected from site M, the open symbols represent earthworms collected from site P, and the crosses represent earthworms collected from site S.

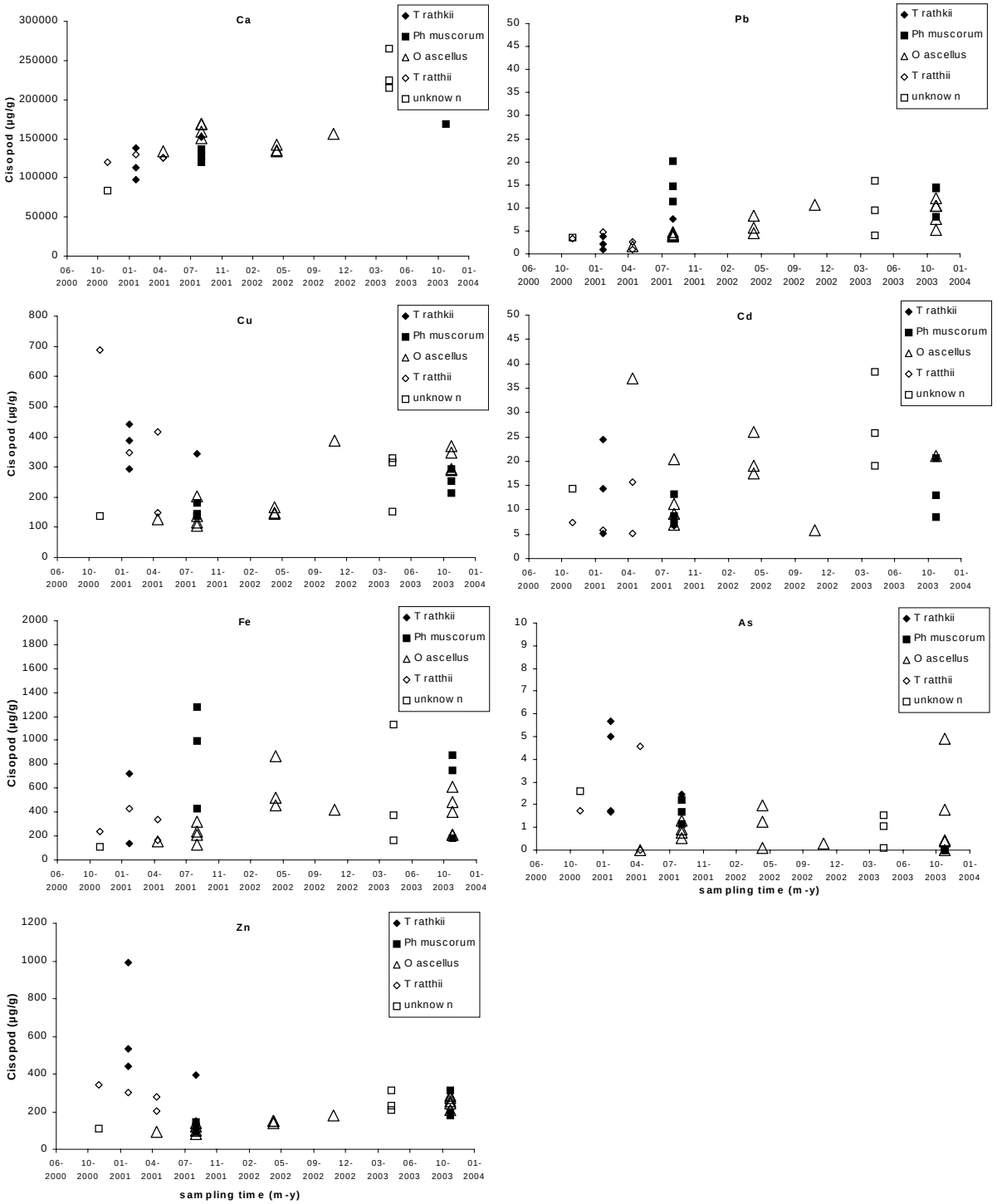

Figure 5: Metal concentrations (µg/g d.w.) in isopod species *Trachelipus rathkii*, *Philoscia muscorum*, *Oniscus asellus* and unknown (=not identified) isopod species sampled from different floodplain sites over time (month-year). In the left column internal concentrations of Ca, Cu, Fe and Zn are shown, in the right column of Pb, Cd and As. The solid symbols represent isopods collected from site M, the open symbols represent isopods collected from site P.

Copper

For Cu, an epigeic earthworm from site M in November 2000 was identified as an outlier and excluded from the dataset (Grubbs test, Q=2.48, n=10), and also an animal from site P in May 2003. All Cu concentrations in earthworms collected in M, P, and S were elevated compared to concentrations found in *L. rubellus* (14.7 ± 5.2 µg/g) from non-polluted soil (Ireland and Richards 1977). Effects on survival of *L. rubellus* are found when internal Cu levels exceed 55 µg/g (Langdon et al. 2001). This concentration was exceeded in earthworms sampled in May 2002 (sites M and P), and in March 2003 (site M). Significant differences (ANOVA, $p < 0.05$) were found within earthworms collected from the three sites. Animals collected from site M in May 2002 had a large biological variation (Fmax > Fcrit), probably explained by internal concentrations close to the effect levels. Because the variance differed greatly between sampling times (see Fmax), analysis of variance could not be performed. Epigeic earthworms collected in February 2001 and May 2002 (site P) also had a biological variation that was larger than the variation between the different groups. Detailed analysis of internal Cu concentrations over time in the different floodplain sites revealed the same pattern as found for Ca concentrations. None of the external soil and pore water characteristics could solely explain the trend in internal Cu concentrations in *L. rubellus*. Even the difference in total metal concentrations (a factor of 7 to 8) between soil M and P compared to site S, was not reflected in the Cu concentrations in *L. rubellus*. Especially in spring and summer the highest internal concentrations were found. Hence, it is suggested that earthworm activity was the predominant factor in the variation of internal concentrations. No significant variation (ANOVA, $p > 0.05$) in Cu levels was found over the season for the endogeic earthworm species.

Also in isopods, Cu body concentrations did not differ between the sampling times (ANOVA, $p > 0.05$). Cu is an essential metal that is actively maintained on a constant level. The isopods kept their internal concentration around 300 µg/g d.w., which is comparable to fixed Cu levels of approx. 340 µg/g d.w. found in isopods collected from unpolluted sites (Dallinger and Prosi 1988). Compared to oligochaetes, Cu body concentrations in isopods were a factor of 10 higher. The metabolically required Cu level is high in isopods, because Cu is a trace metal in the respiratory pigment haemocyanin, whereas oligochaetes require Fe for their respiratory pigment haemoglobin.

Iron

For internal Fe levels, there was a large biological variation within the sampling groups (same time and same site), the average Fe concentrations in *L. rubellus* ranging from 343 µg/g (P, nov 2002) to 5664 µg/g (S, sept 2001). In November 2000 an outlier was found in the samples from site M (Grubbs test, Q=2.58, n=10) that was excluded from further analyses.

Significant variations (ANOVA, $p < 0.05$) with time were found in earthworms collected from site M and site S. Fe concentrations in animals collected in May 2002 and May 2003 of site M were significantly elevated (Tukey, $p < 0.05$) while those collected in November 2000 had lower Fe concentrations. Fe concentrations in animals from site S in September 2001 and May 2001 were elevated (Tukey, $p < 0.05$), while those collected in November 2000 had

lower Fe concentrations. Earthworms sampled at site P showed no significant differences in internal concentrations (ANOVA, $p = 0.066$) over the seasons. Site P is the only site with a litter layer consisting of decomposed material of different trees, herbs and grasses. This may explain the absence of seasonal variations in Fe concentrations of *L. rubellus* inhabiting this floodplain system. Although metal concentrations in litter of site P were not elevated compared to site M (see Table 5), the presence of litter may influence internal metal concentrations in *L. rubellus* indirectly, i.e. through their behaviour. Pore water and total metal concentrations in soil could not explain the differences, neither did inundation frequency nor water quality. DOC concentrations in pore water showed variation with the seasons. DOC level determined in November 2000 was two times higher than in December 2001, which correlates with internal Fe concentrations in the earthworms. However, sorption capacity of metals to DOC levels is hard to detect because reactivity of fulvic and humic acid binding changes with DOC concentration. Hence, the soluble metal pool in soil as a factor has the most predictable power in describing the variation in internal Fe levels.

Fe concentrations in endogeic earthworms were at the same internal level as internal Fe levels in epigeic earthworms. Internal Fe in *A. chlorotica* and *A. caliginosa* varied with time (ANOVA, $p < 0.05$). *A. chlorotica* sampled in May 2001 from site S had significantly higher Fe levels than *A. chlorotica* and *A. caliginosa* species sampled in November 2000 from sites M, P and S (Tukey, $p < 0.05$). This pattern is in agreement with the trend found in the epigeic *L. rubellus.*

Average Fe concentrations in isopods were 452 ± 319 µg/g dry weight and did not differ between sampling times. Fe is an essential metal, regulated at a more or less constant internal level. Compared to oligochaetes, Fe levels in isopods were a factor of 10 lower, which can be explained by the fact that crustaceans do not use Fe as a trace metal in respiratory pigment, as do earthworms (see also the section on copper above).

Lead

Pb concentrations in *L. rubellus* collected from the three floodplain sites were mostly within the range of concentrations (24.7 ± 6.2 µg/g) found in earthworms collected from non-polluted areas (Ireland and Richards 1977). Significant differences over time (ANOVA, $p < 0.05$) were found within earthworms collected from all sites. Differences between individual sampling times were only significant for earthworms collected from sites M and S (Tukey, $p < 0.05$). Earthworms from site P showed no significant variation in internal concentrations over the seasons (Tukey, $p>0.05$). At site M, the animals sampled in March 2003 and December 2001 showed significantly elevated concentrations (Tukey, $p < 0.05$). At site S, the earthworms sampled in September 2001 and December 2001 showed significantly elevated concentrations, while in November 2000 internal concentrations were significantly lower (Tukey, $p < 0.05$). The differences in internal Pb concentrations could partly be explained from DOC concentrations measured in pore water, reflected by the samples taken in November 2000 that had twice as high DOC concentrations than samples taken in September 2001 and December 2001. It should, however, be mentioned that the composition of DOC changed with DOC concentration. Other environmental parameters, such as water quality,

inundation frequency and the soil and pore water characteristics, could not explain variations in Pb levels in *L. rubellus*.

Pb levels in the endogeic *A. chlorotica* and *A. caliginosa* were significantly different between sampling times (ANOVA, $p < 0.05$). Species collected in November 2000 had altered concentrations compared to animals sampled in February 2001 (Tukey, $p < 0.05$). Internal Pb levels were within the range of lowest Pb concentrations found in the epigeic earthworms. The elevated peaks as found for *L. rubellus* were not detected for the endogeic earthworms.

Pb concentrations in isopods were similar to those in earthworms. Variations within sampling times could be found (ANOVA, $p < 0.05$). Isopods collected from site M showed a significantly lower accumulation of Pb in February 2001 (Tukey, $p < 0.05$). At site P, animals had significantly lower internal Pb levels in April 2001 compared to November 2003 (Tukey, $p< 0.05$). None of the environmental parameters could solely explain this variation.

Zinc

Zn concentrations in *L. rubellus* from the sites M, P and S were all elevated compared to levels in animals from non-polluted areas, which ranged between 260 and 925 µg/g (Van Gestel et al. 1992). Significant effects on survival are reported when internal Zn levels exceed 1000 µg/g (Spurgeon and Hopkin 1999). In general, earthworm Zn levels were highest at site P, followed by sites M and S. Significant differences over time (ANOVA, $p < 0.05$) were found within earthworms collected from all sites. At site M, Zn concentrations in earthworms collected in September 2001, March 2003 and May 2003 were significantly higher (Tukey, $p < 0.05$) than in other months and exceeded the level of 1000 µg/g.

Epigeic earthworms sampled from site P showed significantly higher concentrations (Tukey, $p < 0.05$) in February 2001, May 2002 and February 2004 and also exceeded the 1000 µg Zn per gram body weight. Earthworms collected from site S in November 2000 showed significantly lower concentrations compared to animals sampled in other months (Tukey, $p < 0.05$). Endogeic earthworms sampled in November 2000 had significantly lower Zn levels than animals taken in December 2001 (Tukey, $p < 0.05$).

The general trend seems to be that internal Zn concentrations in earthworms were somehow positively correlated with metal concentrations in pore water together with pH. The months February, March, May and September showed significantly higher internal Zn concentrations compared to other months. From pore water properties (e.g. DOC level, pH, anions), it was expected that more metals were available in those months (see Table 3). None of the other environmental factors, such as inundation frequency, presence of litter layers, total metal concentration and soil characteristics, could explain the variations in Zn concentrations.

Zn levels in isopods varied over different sampling times (ANOVA, $p < 0.05$). Isopods collected from site M showed significantly elevated levels in February 2001 (Tukey, $p < 0.05$). However, none of the seasons gave significantly different Zn concentrations in isopods collected from site P (Tukey, $p > 0.05$). Zn accumulation levels in isopods were a factor of 5 lower compared to average Zn concentrations in earthworms. The internal Zn levels in isopods were not elevated compared to those of animals (approx. 329 µg/g) from non-polluted sites (Odendaal and Reinecke 2004).

Cadmium

Cd concentrations in earthworms *L. rubellus* ranged from 12.6 µg/g (S, May 2001) to 150 µg/g (P, Feb 2001 and Nov 2002), by far exceeding levels of earthworms inhabiting non-polluted sites (max up to 26 µg/g) (Van Gestel et al. 1992). Significant differences over time (ANOVA, $p < 0.05$) were found within earthworms collected from the sites M and S. An outlier (Grubbs test, Q=2.21, n=9) from May 2003 of site M was excluded from further analyses. At site M in March 2003 and at site S in February 2001 internal concentrations showed significantly higher levels (Tukey, $p < 0.05$). Cd levels in earthworms collected from site P gave a zigzag pattern over time and were highly variable, therefore no significant differences between the seasons could be found (ANOVA, $p > 0.05$). Earthworms collected in February 2001 and November 2002 from site P exceeded internal Cd levels of 100 µg/g, which are expected to cause significant reduction in the growth of *L. rubellus* (Ma 1983). Cd concentrations in November 2003 were twice as high at site P compared to site M. More importantly, more litter was present at site P compared to sites M and S. This indicates that litter layers may cause the erratic patterns found in epigeic earthworms from site P and their elevated internal levels over the whole year, with extremely high concentrations in February 2001, 2004 and September 2001, May 2002 and November 2000, 2002, 2003. Pore water and total metal concentrations in soil could not explain internal Cd differences neither could inundation frequency nor water quality.

For *A. chlorotica* and *A. caliginosa*, significant differences in Cd levels were found over time (ANOVA, $p < 0.05$), but it was not a zigzag pattern as seen for the topsoil-dwelling earthworm *L. rubellus*. Only endogeic earthworms sampled in November 2000 had significantly lower Zn levels than animals taken in December 2001 (Tukey, $p < 0.05$). Vegetation was obviously not an external factor influencing internal Cd levels in the endogeic earthworms inhabiting the deeper mineral soil layers.

The internal Cd concentrations in isopods collected from site M did not show variations with time (ANOVA, $p > 0.05$), whereas the isopods collected from site P did differ significantly (ANOVA, $p < 0.05$) and had slightly higher internal Cd concentrations. However, when testing Cd concentrations in animals collected from site P over all sampling times, no significant differences could be found (Tukey, $p > 0.05$). Striking is that although isopods are litter-dwellers, their internal Cd level does not show a zigzag pattern as shown for the epigeic earthworms. As seen in Table 4, Cd concentrations in litter did not differ much between site M and P, except for litter collected in November 2003. This difference could not be found in internal isopods levels. It may be suggested that the presence of litter does not have such an impact on the Cd accumulation in isopods as seen for *L. rubellus*, rather Cd concentration in the litter layer is important for Cd accumulation in isopods.

Arsenic

For As levels, a large biological variation existed within the sampling groups (same time and same site), with average As concentrations in *L. rubellus* ranging from 4.71 µg/g (S, nov 2000) to 58.4 µg/g (P, nov 2002). In November 2000 at sites M and S, outliers were found (Grubbs test, Q=2.26, n=10 and Q=3.13, n=15), those data were excluded from further

analyses. Internal concentrations were elevated compared to As levels (max 12 µg/g) found in earthworms collected from non-polluted sites (Van Gestel et al. 1992). Internal As concentrations in earthworms from site M were positively correlated to body weight (ANCOVA, $p = 0.004$). In general, accumulation in earthworms was highest during autumn and winter, and body weight contributed to temporal fluctuations in internal metal concentrations. Earthworms from sites P and S showed no significant differences in As concentrations over time. A relationship between internal As levels and environmental parameters determined could not be established.
As accumulation in the endogeic earthworm species was approximately 20 µg/g and did not show variations in time (ANOVA, $p > 0.05$).
For all isopod species, As accumulation was very low and no variation between sampling times was detected. No positive correlation of As levels with body weight was found for isopods, although especially isopods do have the physiological possibility (based on detoxification mechanisms) to accumulate some contaminants during their entire lifespan (Hopkin 1989).

Chromium
Cr concentrations in *L. rubellus* showed large biological variation within the sampling groups (same time and same site). In November 2000, site M, and February 2004 site P, outliers were found (Grubbs test, Q=2.57, n=10 and Q=1.67, n=5), those data were excluded from further analyses. Cr concentrations in earthworms sampled from all three sites were elevated compared to levels (max 3 µg/g) found in earthworms from non-polluted areas (Van Gestel, 1992). Although the internal levels were elevated, *L. rubellus* sampled from the floodplain soils are not at risk from Cr intoxication, because internal Cr levels were much lower than the critical effect concentrations derived from toxicity experiments. E.g. EC_{50} level for cocoon production of *Eisenia fetida* was 890 mg/kg d.w. for Cr (III) (Lock and Janssen 2002), which is way higher than the soil concentrations in the floodplain soils concerned (Table 2). Significant differences over time (ANOVA, $p < 0.05$) were found within earthworms collected from the sites M and S. Earthworms from site P showed a too large biological variation (Fmax) for testing variation between the sampling times. At site M, internal concentrations in March 2003 and December 2001 were significantly higher (Tukey, $p < 0.05$). Earthworms from site S had significantly elevated Cr levels in September 2001 and May 2001 (Tukey, $p < 0.05$). None of the environmental parameters determined could explain the differences, not even inundation, although from literature it is known that speciation of Cr in soil is strongly influenced by redox-conditions (Bourg 1995). It should be noted that Cr concentrations in porewater were slightly higher than the detection limit.
Cr concentrations in *A. chlorotica* and *A. caliginosa* were up to approx. a maximum of 15 µg/g and did not vary over time (ANOVA, $p > 0.05$). Internal Cr concentrations in endogeic earthworms were comparable to the lowest Cr concentrations found in *L. rubellus*.
In all isopods, Cr concentrations were near the detection limit, indicating that Cr is hardly taken up by isopods.

2.4 Synthesis

The bioavailability of metals to soil-invertebrates may vary depending upon the chemical and physical modifying factors present in the soil and the biology of the species.
Within the epigeic earthworm species *L. rubellus*, significant differences in internal metal concentrations were found, although none of the measured fluctuating environmental factors could solely explain the variations. Only a few relationships between the variation in internal concentrations and single external descriptors were found, Table 5 provides an overview of the relationships.

Table 5: Relationships between variations in internal metal concentrations in earthworms and external factors. Worm activity includes e.g. reproduction and burrowing behaviour.

Metal	Determining factor
Epigeic earthworm *Lumbricus rubellus*	
Pb	Soluble metal levels in soil
Fe	Soluble metals in soil, presence of litter layer, worm activity
Cd	Presence of litter layer
Zn	Pore water concentration, pH
As	Body weight (only in site M)
Ca	Worm activity
Cu	Worm activity
Endogeic earthworms *Allolobophora chlorotica* and *Aporrectodea caliginosa*	
Fe	Worm activity
Zn	Pore water concentration, pH

It can be stated that physico-chemical environmental factors have an impact on the variation in metal concentrations in biota. Location-specific differences in Zn, Cd, As, and Cr concentrations in *L. rubellus* were found, whereas Ca, Cu, Fe and Pb levels had a similar pattern in time over the three different floodplain sites. For both endogeic earthworm species, location-specific differences in internal Pb and Cd concentrations could be found, whereas all other internal metal levels and internal As levels were similar in time over the three different floodplain sites. For isopods, no location-specific accumulation differences were detected, except for internal Cd levels.

Unlike for the epigeic earthworm, variation of internal concentrations in the endogeic earthworms over time was not found for all metals. Hence, the ecology of earthworm species has an impact on the internal metal level, explained by the difference that epigeic species preferably burrow and feed in top layers of the soil while endogeic species prefer the deeper mineral soil layers. Ma (2004) states that in case of field-sampled earthworms, it is important to identify the species according to their pedo-ecological classification (according to Bouché 1977).
For isopods, hardly any differences in internal concentrations could be found over the seasons. Although isopods mainly feed on organic matter, the presence of litter consisting of decomposed plant material did not cause structural differences in metal accumulation in animals collected from site M and site P. It should be noted that metal concentrations in the litter layer consisting of decomposed plant material (site P) and litter consisting of grasses and

reed – and therefore less easy to digest for isopods (site M) did hardly differ (Table 4). Therefore hardly any differences in metal levels in isopods inhabiting the two different floodplain soils (site M and P) were detected and metal concentrations did not show large differences over the seasons. Although especially isopods are known to grow almost their entire life-span and have a storage capacity to accumulate contaminants, metal levels were not related to body weight like in the epigeic earthworms.

The species-specific metal accumulation patterns were obvious and showed that ecological factors, especially the organism's habitat and animal physiology, have an impact on bioaccumulation. Physico-chemical characteristics appeared to have an impact on bioaccumulation as well, however, neither a physical nor a chemical environmental factor could solely explain the differences in bioaccumulation. Nevertheless, it should be noted that local variations in metal concentrations and for instance the rate of metal supply from the solid phase to the soluble phases are very difficult to measure in the field. In floodplain soils that are characterized by their low available metal concentrations, it is not unlikely that depletion of the pore water occurs (Pinheiro et al. 2004) in the direct surroundings of the earthworms. The desorption-limited pore water pool has strong consequences for bioavailability (Vink 2002) and may be partly responsible for the variation in metal concentrations in animals. Especially location-specific differences over time may be explained this in way. Therefore, earthworms and isopods can be seen as the integrator of all factors influencing metal bioavailability. No conclusive statement could be made on the environmental factors having an impact on the variation in internal metal concentrations in the earthworms and mechanistically based studies in the laboratory need to be executed.

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Chapter 3

Oral sealing using glue; a new method to distinguish between intestinal and dermal uptake of metals in earthworms

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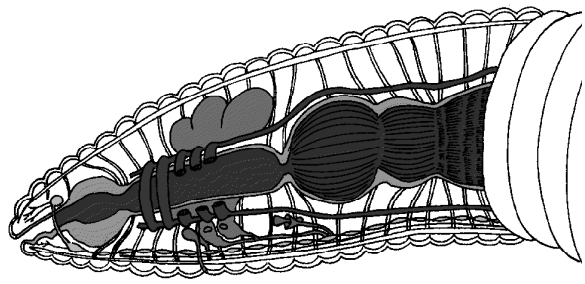

Chapter 3
Oral sealing using glue; a new method to distinguish between intestinal and dermal uptake of metals in earthworms

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Abstract

Earthworms may take up chemicals from soil and pore water, both through their skin (dermal) and by ingestion (oral). It remains unclear, however, what the relative importance of these pathways is. To assess bioavailability of pollutants in soil to earthworms, it is necessary that the contribution of each pathway is known. *Lumbricus rubellus* were sealed by means of medical histoacryl glue, to block ingestion of soil particles and pore water. For 6 d, these earthworms showed good survival and vitality and no soil ingestion was found. Equal metal uptake was found by sealed and unsealed earthworms exposed to an inert sand matrix continuously flushed with contaminated water. Therefore, pore water uptake via ingestion contributes little to metal accumulation. Uptake rates of Cd, Cu and Pb in sealed and unsealed earthworms exposed to two contaminated field soils were similar. Uptake and elimination kinetics of Zn were significantly lower in sealed earthworms exposed to one of the two field soils. Body concentrations of Cu and Pb could be completely attributed to the dermal route. For internal Cd and Zn concentrations, however, 0% - 17% and 21% - 30% respectively were derived from ingestion. It is concluded that for metals the dermal route is the uptake route of importance. The sealing method described here may be useful in a variety of earthworm nutrition and contamination-effect studies.

3.1 Introduction

At present, risk assessment of pollutants in soil receives much attention. Earthworms are ecologically important for their role in soil health through their abundance, their role in decomposition and soil texture improvement, and their key position in terrestrial food chains (Edwards and Lofty 1972). For these reasons, earthworms are recognized and representative test organisms for ecological risk assessment (Eijsackers 1997). Earthworms may be exposed to contaminants in various ways. Firstly, living in the soil they are in direct contact with soil pore water. The biochemical composition of the earthworm's cuticle is well investigated, and is extremely tolerant to water uptake and loss (Wallwork 1983). Considerable exchange of water occurs across the body wall (Laverack 1963). Secondly, uptake of pollutants adsorbed to solid particles in the soil plays a role, due to the earthworm's burrowing behaviour and their digestion of organic soil constituents including leaf litter (Edwards and Lofty 1972). Following ingestion, mucus is mixed in with the soil and food. Gut conditions may decrease the number of metal binding sites in the soil and food, by changing the chemical composition of gut liquids and by excreting digestive enzymes (Wallwork 1983).

To assess the risks of soil pollution, such as metals, it is important to know the pathways along which pollutants will enter the body. Nowadays soil protection is based on total metal concentrations in soil. Many studies, however, indicate that chemical speciation of metals should be considered and that the soluble fraction of a contaminant gives a better indication of risk than the total concentration. Chemicals taken up via the dermal route can be directly related to the pore water concentration or a specific chemical species in the pore water. Via ingestion, solid particles and soil solution can be taken up, but uptake of chemicals through this route cannot directly be explained from pore water concentrations or soil properties.
Many studies provide evidence that chemicals have to be in a dissolved state to be bioavailable to earthworms (Belfroid et al. 1993, Spurgeon and Hopkin 1996, Peijnenburg et al. 1999, Osté et al. 2001, Saxe et al. 2001). These are empirical studies, mainly based on correlations between several external pollutant pools and internal concentrations in earthworms. Not in all cases, however, pore water is the sole contributor of pollutant accumulation (Belfroid et al. 1994, Marinussen et al. 1997). Uncertainties still exist in quantifying uptake routes for metals, due to the lack of an experimental method that accurately determines the contribution of exposure routes. To focus on the impact of typical soil processes such as adsorption/desorption and precipitation/dissolution, research with earthworms has sometimes been performed in aqueous environments (Kiewiet and Ma 1991, Janssen et al. 1996). A disadvantage of these studies is that elimination rates and vitality of earthworms in such aqueous systems are not representative of that in a soil matrix.
We have investigated a novel method to distinguish between uptake routes of metals in earthworms. The validity of the method was demonstrated by means of two types of metal exposure experiments, namely in an inert sand matrix flushed with contaminated water and in two polluted field soils. Metal uptake via the skin (dermal) and by ingestion of solid and soluble fractions (oral) was quantified by means of uptake and elimination kinetics.

3.2 Materials and method

Earthworm collection and conditioning

Earthworms of the species *Lumbricus rubellus* were collected from a non-polluted forest soil in Lepelstraat, The Netherlands. The animals were kept in the laboratory for 2 months in this soil with poplar leaves *Populus x canadensis* for food. Before use in the experiments the worms were kept on filter paper for 48 h to void gut contents. Initial individual fresh masses of the earthworms were 0.4 – 0.7 g. Incubation conditions were maintained at 80% relative humidity, 12 ± 1°C and permanent illumination with intensity 2000 lux.

Sealing the mouth parts of earthworms

The methodology to exclude earthworms from oral uptake is by using a medical glue. Histoacryl glue (Braun aesculap, Germany), made of enbucrilate, was developed for medical purposes, e.g., to seal human tissues. The glue is odorless and not corrosive. Histoacryl glue is easily applied to the organism's tissues by dipping the earthworm's mouth in the glue. The glue dries within 30 s. In this way, oral uptake is excluded. Liquid or mucus must be removed before applying the glue to the worm's mouth, since this will inhibit the drying process. Figure 1 shows an earthworm that was sealed with histoacryl glue.

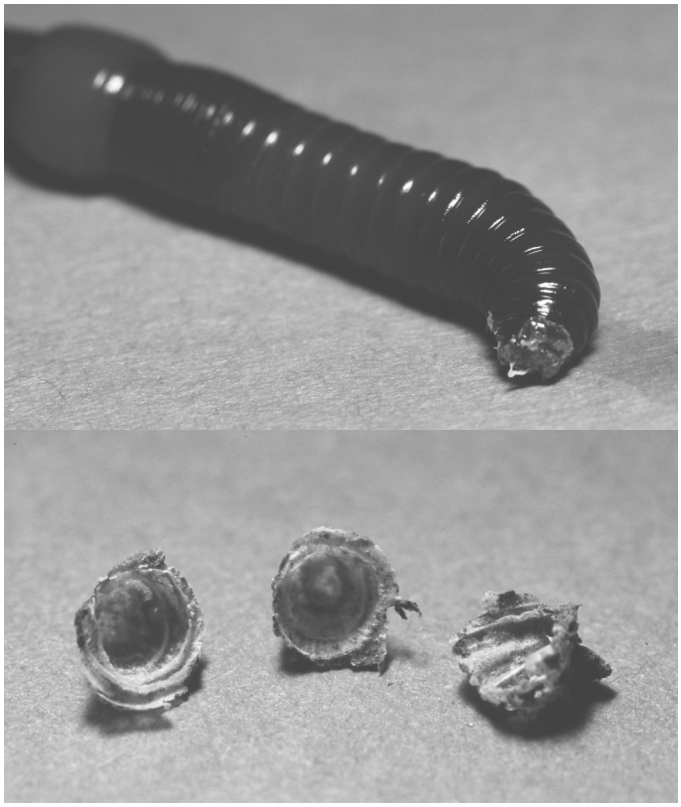

Figure 1: Photograph of a sealed earthworm, and glue-caps remained after sacrificing the earthworms

Water exposure experiment

Sixteen orally sealed and 24 unsealed organisms were exposed in an inert sand matrix. Five unsealed earthworms and 6 sealed earthworms were taken for initial metal concentration determination. In the inert sand matrix no food was available. Therefore, quartz sand (200 µm

to < 2 mm) was used as an inert matrix by rinsing the sand with a 0.7 M HNO_3-solution, to remove organic matter and reactive Fe and Mn components. To neutralise acidity, the sand was dried and washed afterwards several times with 10 mM $CaCl_2$ and once with 10 mM $CaCO_3$, the amount depending on the pH measured. Care must be taken with precipitation of excess $CaCO_3$ and the forming of new carbonate adsorption sites. Portions of (375 g) dry sand were placed in 1-l glass jars and continuously flushed with natural contaminated river water at a rate of 0.8 ml min^{-1}. Four earthworms were transferred to each jar. After 1, 2, 4 and 6 d of exposure, one jar of each treatment was sacrificed and all earthworms were recovered. Jars containing unsealed earthworms were additionally sampled after 8 and 13 d.

Soil exposure experiment

In a second experiment, a total of 78 clitellate earthworms were exposed in two metal-contaminated field soils under laboratory conditions. Soil M was collected at Ruitersplaat, soil P at Lage Hof, both in a contaminated flood plain area south of Werkendam, The Netherlands. Thirty-eight earthworms were sealed to prevent oral uptake using medical glue as described above. Portions equivalent to 400 g dry soil were added to 1.5-l jars. Four earthworms were placed in each jar. Vitality of the animals was monitored up to 6 d for the sealed worms and 9 d for the unsealed ones. At each sampling time, one test jar per treatment was sacrificed. During the experiments, burrowing behaviour, healthy appearance and mortality of the earthworms were observed.

Treatment of earthworms after exposure

After collection, animals were allowed to void their gut for periods up to 48 hours. Each individual was scored for its ability to feed, focusing on the earthworm's mouth and its gut content. Faeces (gut pellets) were scored and weighed to see whether the sealing method was working well. Fresh weight of the earthworms was determined after rinsing and blotting the animal on a filter paper. Subsequently, the organisms were killed by immersion for a few seconds in boiling water. Upon this treatment, glue released from the earthworms' body, and a glue-"cap" was left behind (see Figure 1). Removing the glue-caps prevents possible interferences in chemical analyses. Dry weight of each individual was measured after at least 48 h of freeze-drying. Some sealed and unsealed individuals were ashed at 550°C, to check for the absence of soil and sand particles.

Metal analyses in water, soil and earthworms

Aqua regia digestion was performed to obtain actual total metal concentrations in soil. Pore water was sampled by using a permeable soil water sampler (Rhizon SMS-MOM, The Netherlands), which was placed in the sand matrix or the soil, and analysed for soluble metal concentrations and DOC content.

Lyophilized earthworms were digested individually in 7 ml 14.9 M HNO_3-solution (70% pro-analysed Baker) and 3 ml demineralised water. At least four organisms were analysed at each exposure time to obtain insight in the biological variation among individuals. Metal concentrations in the soil, earthworm and pore water digests were determined using ICP-MS.

Dolt-2 (certified by the Community Bureau of Reference, BCR, Brussels, Belgium) was used as biological reference material. Recoveries were typically between 90 and 110%.

Data treatment

A one-compartment model was used to analyse the development of earthworm body concentrations with time, and to calculate uptake and elimination rate constants. The increase of internal concentrations during exposure to an environmental concentration C_e is written as

$$C_{iworm}(t) = C_i(0)e^{-k_2 t} + \frac{k_1 C_e}{k_2}.\left(1 - e^{-k_2 t}\right) \quad [1]$$

with: $C_{iworm}(t)$ = metal concentration in the earthworm (µg g^{-1} dry weight), t = time (d), k_1= uptake rate constant (d^{-1}), C_e = external concentration (either in µg g^{-1} dry soil or µg l^{-1} pore water), k_2 = elimination rate constant (d^{-1}). $C_i(0)$ is the initial body concentration, which is measured in the animals before exposure and is assumed to participate in all equilibration processes within the organism.

In the experiment with the sand matrix, the concentrations of Cd and Zn in water decreased with time. Assuming first order dissipation kinetics, the external concentration was calculated applying equation 2:

$$C_e(t) = C_e(0)e^{-k_0 t} \quad [2]$$

with: $C_e(0)$ = initial external concentration (µg l^{-1}), t = time (days), k_0=rate constant for loss of the metal in the water (d^{-1}). In this case, uptake and elimination kinetics were derived as described by Widianarko and Van Straalen (1996)*:*

$$C_{iworm}(t) = C_i(0)e^{-k_2 t} + \frac{k_1 C_0}{k_2 - k_0}.\left(e^{-k_0 t} - e^{-k_2 t}\right) \quad [3]$$

Values of k_1 and k_2 were estimated applying equation [1] and [3], using the non-linear module of Systat 10.0. Significance of uptake and elimination rates for sealed and unsealed earthworms was compared using a generalized likelihood ratio test (Sokal and Rohlf 1969). Two-way ANOVA statistics were applied to test for the difference in the development of internal concentrations with time (as a covariable) between treatments (factor).

3.3 Results

Water and soil characteristics

Moisture content of the sand in which the earthworms were exposed was 26% on a dry weight basis (w/w), which is above the water holding capacity (WHC). In the field soil M, moisture content was 67% w/w, which corresponds to 60% of WHC, and in the field soil P it was 55%

w/w (74% of WHC). The exposure conditions and soil and pore water characteristics are given in Table 1.

Table 1: Characteristics of the sand matrix and the two field soils (M and P) in which the earthworms were exposed

	Sand	M	P
DOC (mg l^{-1})	2.5	29	22
Redox (mV)	210-240	249-271	240-260
pH-$CaCl_2$	8.22	7.93	7.82
Totaal C (%)	n.d.	16.9	6.4
Total metal concentrations			
Cd (µg g^{-1})	ND	14.1	4.46
Cu (µg g^{-1})	ND	331	79
Pb (µg g^{-1})	ND	554	177
Zn (µg g^{-1})	ND	2092	732
Soluble metal concentrations			
Cd (µg l^{-1})	0.9	2.6	0.9
Cu (µg l^{-1})	3.2	43	19
Pb (µg l^{-1})	0.2	0.2	0.3
Zn (µg l^{-1})	9	96	66

ND = not determined

Organism performance and metal accumulation

Lumbricus rubellus exposed to the sand matrix and to the two field soils were all in good condition upto an exposure time of 6 d. This was determined by visual inspection of the worms, e.g., color, activity and constriction of the body. The burrowing capacity of sealed and unsealed earthworms was similar and they all disappeared into the sand or soil. No obvious visual differences in activity and locomotion were found, although initially the sealed earthworms needed approximately 1 h to begin burrowing.

Survival of sealed and unsealed earthworms (Table 2) was not significantly different (ANOVA; $P>0.05$, n.s., for both soils and the sand matrix). Adverse effects, usually starting with constriction of the body, were found in six out of 121 individuals. Earthworm wet and dry weights (Table 2) in the different treatments did not significantly differ during the exposure time. The ratio dry-to-wet weight of the earthworms was on the average (±SD) 5.58 ± 0.95 (n= 121).

Sealed earthworms could only be kept on filter paper for 36 h, since they showed a decline in vitality. This is possibly due to starvation. The amount of excreta produced on filter paper by sealed earthworms kept in quartz sand was 34.8 ± 7 mg dry weight (n=6), while unsealed animals had a four times higher (T-test; t-value = 7.08, $P<0.05$) voiding content of 150.6 ± 40 mg dry weight (n=6). Residue after ignition (ash to dry weight basis) of the sealed worms was 280 ± 200 mg (n=3). Unsealed earthworms had an ash content of approximately 550 ± 110 mg (n=5). Only one out of all sealed animals had soil or sand particles in its gut. This animal was excluded from further analyses.

Table 2: Survival, body weights and dry weight content of sealed and unsealed *Lumbricus rubellus* exposed for different periods in a sand matrix and two field soils

	sealed earthworms				unsealed earthworms			
time (days)	Initial number	Surviving number	dry w. (mg)	% dry of wet	Initial number	Surviving number	dry w. (mg)	% dry of wet
sand matrix								
0	6	6	110± 31	5.0± 0.5	5	5	107± 55	4.4± 0.6
1	4	3	84± 6	3.9± 0.8	4	4	94± 47	4.8± 1.3
2	4	3	62± 10	4.8± 0.5	4	3	104± 66	4.4± 2.0
4	4	3[a]	79± 56	5.1± 0.1	4	3	64± 9	6.6± 0.3
6	4	3	94± 83	4.8± 1.2	4	4	71± 16	5.2± 1.0
8	ND	ND	ND		4	3[a]	123± 118	3.9± 2.5
13	ND	ND	ND		4	4	59± 33	4.9± 1.8
field soil M								
0	3	3	84± 35	5.7± 0.1	4	4	64± 24	7.3± 1.8
1	4	4	93± 29	4.9± 0.3	4	4	64± 12	5.6± 0.7
2	4	4[a,b]	90± 9	4.8± 0.3	4	4[a]	60± 9	5.9± 0.7
4	4	4	100± 28	4.7± 0.6	4	4[a]	76± 14	5.4± 0.7
6	4	4	79± 16	4.9± 0.5			.	
9	ND	ND	ND		4	4	70± 19	5.8± 1.5
field soil P								
0	3	3	84± 35	5.7± 0.1	4	4	64± 24	7.3± 1.8
1	4	4[a]	97± 23	4.9± 0.3	4	4	56± 10	5.4±0.7
2	4	4	81± 12	4.7± 0.2	4	4	49± 7	6.1±0.7
4	4	4	97± 24	4.7± 0.2	4	4	79± 27	5.3± 0.8
6	4	3	95± 15	4.8± 0.2				
9	ND	ND	ND		4	4[c]	56± 6	6.4±1.5

ND = no data determined, [a] = one animal showed visual adverse effects, [b]= one animal with soil in the gut, [c] = one animal escaped during gut voiding time.

Metal accumulation by sealed and unsealed earthworms when exposed in an inert sand matrix with natural contaminated water (Fig 2 (A)) increased with time up to 4 d, followed by a decline that is due to the decreasing metal concentrations in the water.

In case of Cu and Pb, the data are rather scattered and there was no clear uptake pattern. No significant difference in metal accumulation between sealed and unsealed animals was found in these flow-through systems (ANOVA; Cd, p=0.138, n=39, n.s.; Pb p=0.884, n=40, n.s.; Cu p=0.503, n=39, n.s., Zn p=0.369, n=39, n.s.; interaction term for all metals n.s.). Metal accumulation kinetic parameters and the rate constants for dissipation of Cd and Zn from the water phase are given in Table 3. For Pb, only a very poor fit was obtained, and uptake curves are therefore not shown in Fig 2. Uptake and elimination rate constants for all metals did not significantly differ between the two treatments ($\chi^2_1 < 3.84$, n.s.). This means that pore water uptake via ingestion contributes negligibly to metal accumulation.

The development of internal metal concentrations with time for sealed and unsealed earthworms exposed to soil P (Figure 2 (B)) and soil M (not shown) showed a similar pattern. Body concentrations for all metals increased with exposure time. Measured initial metal concentrations in the earthworms were 5.6 ± 1.7 µg Cd g^{-1}, 8.8 ± 1.5 µg Cu g^{-1}, 22.6 ± 5.6 µg

Pb g^{-1}, 457 ± 134 µg Zn g^{-1} dry weight (n=7), initial concentrations in sealed worms were not significantly different (T-test, P<0.05) and amounted 4.0 ± 0.1 µg Cd g^{-1}, 9.3 ± 0.3 µg Cu g^{-1}. 12.4 ± 8.3 µg Pb g^{-1}, 300 ± 49.5 µg Zn g^{-1} dry weight (n=2).

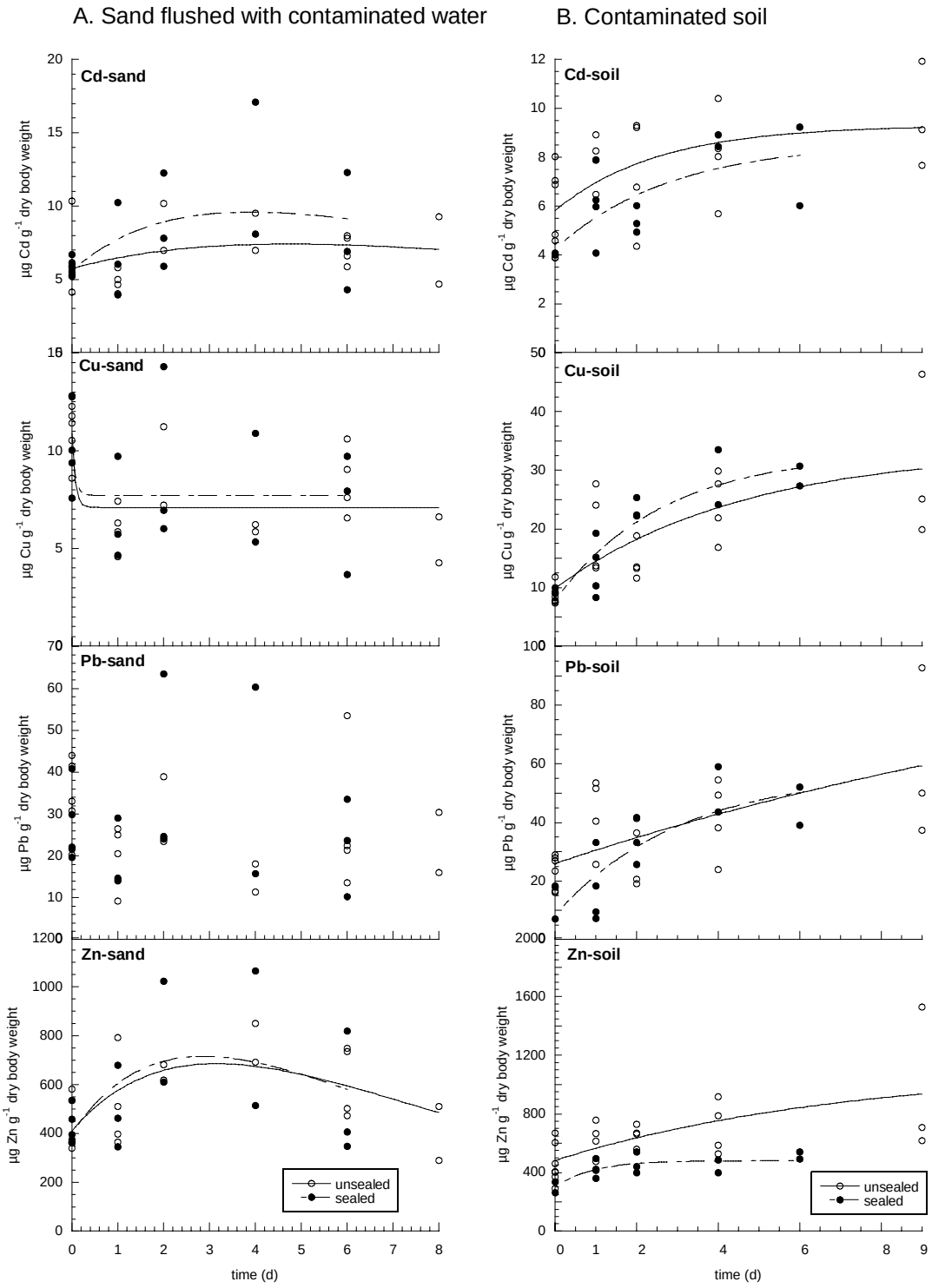

Figure 2: Uptake of Cd, Cu, Pb and Zn by *Lumbricus rubellus*, sealed or unsealed, upon exposure to a sand matrix flushed with contaminated water (A) or contaminated soil P (B). Lines are fitted according to equations 1 (all metals in soil and Cu in sand) or 3 (Cd and Zn in sand). For Pb in sand, no proper fit could be obtained. Note differences in scales of vertical axes.

In soil P, internal Zn concentrations (Figure 2 (B)) were significantly lower in sealed earthworms than in earthworms exposed via both the dermal and oral route (ANOVA; F= 11.57, p=0.002, n=35), but there was a significant interaction between time and treatment (p=0.005), showing that this difference increases with time. For the other metals, internal concentrations in sealed and unsealed earthworms did not significantly differ (ANOVA; p > 0.05, n.s.; interaction term n.s.). Earthworms exposed to soil M gave similar results, although here also Cd body concentrations were significantly elevated in unsealed earthworms (ANOVA; Cd, F=5.22, p=0.028, n=34; Zn, F= 5.18 p=0.03, n=34). Thus, oral uptake plays a role in Zn accumulation, and may also contribute to Cd accumulation in earthworms. For Cu and Pb, total uptake can be ascribed to the dermal route. Exposure up to 6 d gave 30% and 21% less Zn uptake in sealed earthworms as compared to unsealed earthworms exposed to soil P and M. Initial Cd uptake in worms exposed to soil M was scattered, with average internal Cd concentrations of sealed earthworms being 83% of the body concentrations measured in unsealed earthworms. Metal uptake and elimination rate constants and estimated initial body concentrations in both field soils are given in Table 3.

Analysing the kinetics data, it appeared that uptake rates of Cd, Cu, Pb and Zn in sealed and unsealed earthworms were not significantly different for soil P ($\chi^2_1 < 3.84$, (n=35), soil M; $\chi^2_1 < 3.84$ (n=34)). However, Zn uptake rates were significantly higher in unsealed compared to sealed earthworms exposed to field soil M ($\chi^2_1 = 5.28$, $p < 0.025$ (n=34)). This means that uptake by ingestion is negligible for Cd, Cu and Pb, and may therefore entirely be attributed to dermal exposure. Elimination rate constants of Cd, Cu and Pb for both treatments in both soils were not significantly different ($\chi^2_1 < 3.84$), however, the Zn elimination rate constant of unsealed earthworms exposed in soil M was significantly higher ($\chi^2_1 = 6.04$, $p<0.025$).

Table 3: Uptake and elimination rate constants of metals accumulated by sealed and unsealed earthworms, exposed in a sand matrix flushed with contaminated water or two contaminated field soils. k_0 = rate constant for metal loss (d^{-1}), C_0 = initial concentration in the earthworm ($\mu g\ g^{-1}$), k_{1total} = uptake rate constant based on total metal concentrations in the soil (d^{-1}), k_{1pw} = uptake rate constant based on pore water metal concentrations in the soil or sand matrix (d^{-1}), SE_1 = standard error of the uptake flux ($\mu g\ d^{-1}$) which is defined as k_1 C with k_1 either based on k_{1total} or on k_{1pw} , k_2= elimination rate constant(d^{-1}), SE_2 is the standard error of k_2

soil	Metal	k_0	Sealed earthworms							Unsealed earthworms						
			C_0	k_{1total}	k_{1pw}	SE_1	k_2	SE_2	R^2	C_0	k_{1total}	k_{1pw}	SE_1	k_2	SE_2	R^2
Sand matrix	Cd	0.25 ± 0.05	5.49	-	99	1.28	0.22	0.16	0.88	5.73	-	2.25	0.96	0.18	0.11	0.93
	Cu	-	3.79	-	36.5	ND	16.1	1.85	0.18	4.82	-	29.8	2.12	14.3	ND	0.46
	Pb	-	25.6	-	109	ND	0.81	ND	0.01	33.9	-	2597	ND	24.2	ND	0.16
	Zn	0.06 ± 0.04	400	-	100	ND	0.32	ND	0.31	401	-	100	ND	0.27	ND	0.38
Field soil M	Cd	-	3.96	0.30	1.61	2.67	0.44	0.35	0.97	5.57	0.26	1.39	1.42	0.26	0.13	0.97
	Cu	-	8.74	0.08	0.63	20.4	0.20	0.32	0.88	5.69	0.19	1.47	19.6	0.65	0.25	0.91
	Pb	-	2.69	0.02	350	60.7	0.46	0.57	0.76	16.5	0.15	429	38.3	0.64	0.34	0.86
	Zn	-	295	0.19	4.18	271	0.54	0.44	0.96	450	0.36	7.77	282	0.81	0.34	0.98
Field soil P	Cd	-	4.23	0.67	3.26	2.74	0.35	0.40	0.96	5.81	0.82	3.99	2.98	0.40	0.36	0.95
	Cu	-	7.78	0.16	0.65	4.85	0.38	0.20	0.97	9.85	0.09	0.38	4.06	0.22	0.18	0.90
	Pb	-	8.33	0.10	74.1	9.79	0.32	0.26	0.93	25.3	0.04	27.6	8.82	0.07	0.20	0.88
	Zn	-	301	0.71	7.81	279	1.08	0.60	0.99	482	0.21	2.32	167	0.14	0.22	0.92

ND = not determined.

3.4 Discussion

Metal risk assessment develops in a way that effects are related to soluble metal concentrations rather than to total amount of metals in the soil (Allen 1997). When dermal uptake is the only route along which metals are accumulated, exposure and effects may indeed be well explained from soluble concentrations in the soil. If however, oral uptake plays a significant role in metal accumulation, such a relationship with soluble concentrations may not be straightforward. After oral uptake, digestion processes actively change the soil-solution distribution of metals. For a proper risk assessment, it is therefore important to know the relative contribution of different uptake routes.

The experimental data presented in our study are the first to give experimental evidence on the contribution of different exposure routes in earthworms.

Other studies found in the literature are always based on empirical regressions between several metal pools in soil and earthworm body concentrations. The sealing method is suitable to determine bioaccumulation of chemicals in earthworms due to different exposure routes for up to 6 d. In a study on the calciferous glands of *Lumbricus rubellus* by Piearce (1972), earthworms were orally ligatured with a glass dumbbell that was tightened around the mouth with a cotton robe. After 18 h, 20 out of 48 worms had retained their ligatures (Piearce 1972). Sealing earthworms with medical glue does not require any extreme dexterity. The method proved to be suitable for *Lumbricus rubellus*, but may be more difficult to apply on other earthworm species, because mucus can interfere with the drying process of the glue.

The vitality of sealed earthworms was not significantly different from unsealed ones, up to 6 d of exposure. Although the medical glue dissolves in water after a while, in most cases the glue was retained on the earthworms throughout the experiment. In some cases the glue detached prematurely, and in these cases the mouth-segment was disrupted; the tissue was bleached and the prostomium deformed in such a way that eating soil particles probably was not possible anymore. This statement was supported by the results on the check for gut contents by ashing earthworms. Metal uptake by earthworms that retained and maintained their glue-cap was similar and so the disrupted mouth-segment probably also excluded the possibility of drinking pore water.

The sand matrix used to quantify the influence of soluble fractions on uptake through the skin (dermal) and drinking (oral), contained hardly any sorption sites due to acid treatment. This matrix allows earthworms to stay in a good condition and provides a possibility to determine accurate elimination rates. In comparison, Kiewiet and Ma (1991) found adverse effects after only 24 h exposure of earthworms in water. In an aqueous medium, the mucus layer will be damaged and the earthworms may have problems to maintain their osmotic pressure (Oglesby 1978). Additionally, the elimination is not natural in such systems, due to the lack of a solid matrix. The same problems with elimination rates were recognised in a study on cesium uptake in earthworms described by Janssen (1996). Earthworms move through the soil and chafe their skin to solid particles. The thickness of the mucus layer is maintained by excretion of fluid, which may also be a way to eliminate metals from the body.

No significant difference in uptake rate constants could be found for Cd, Cu and Pb in sealed and unsealed earthworms, but Zn uptake was significantly lower in sealed earthworms compared to unsealed individuals. For Zn, 21-30% of the uptake could be attributed to the oral route. Similar results were found by Saxe et al. (2001), who derived an empirical model that determined the contribution of oral and dermal exposure routes to metal uptake for *Eisenia andrei*. Their results indicated that for Cd, Cu and Pb uptake was for up to 96% due to dermal exposure and that 82% of internal Zn concentrations were due to this route.

Our experimental data show that elimination rate constants for the different metals are not significantly different between sealed and unsealed earthworms. Therefore, it is suggested that blocking the earthworm's mouth does not affect metal elimination, although the possibility of excreting particles and thereby simultaneously eliminate metals is excluded.

Our experimental data support models that showed that soluble metal pools are best descriptors of metal accumulation in earthworms (Spurgeon and Hopkin 1996, Peijnenburg et al. 1999). Additionally, a model made by Marinussen and Van der Zee (1996) demonstrated that Cd uptake is largely affected by pore water but that the pore water fraction alone is not a good descriptor of metal concentrations in earthworms. Osté et al. (2001) found that a metal-binding substrate, such as MnO_2, reduced Cd uptake in *L. rubellus.* Nevertheless, the effect of this adsorbent on pore water free Cd concentrations was much smaller. Our results agree with these qualitative findings. Our research on the contribution of different uptake routes was first aimed as an illustration of the suitable use of sealing techniques. The soils in which the tests were performed are carbonate rich with a relatively high pH. It is possible that the impact of the two uptake routes will depend on soil conditions. Osté et al. (2001) demonstrated that taking the competition of protons and cadmium on the earthworm's body surface into account gave a good description of Cd uptake. By using several extraction methods, they suggested a predominance of pH-independent intestinal uptake of Cd. In theory, for complexed metals oral uptake seems to be most relevant, as digestion of ingested soil material may increase bioavailability. In this study, we used clayey soils, rich in carbonate, therefore having a strong metal binding capacity. Nevertheless, our results clearly demonstrate a predominance of dermal uptake over oral uptake. Therefore, we argue that dermal uptake is the most important uptake route.

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Chapter 4

Biphasic elimination and uptake kinetics of Zn and Cd in the earthworm *Lumbricus rubellus* exposed to contaminated floodplain soil

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Chapter 4
Biphasic elimination and uptake kinetics of Zn and Cd in the earthworm *Lumbricus rubellus* exposed to contaminated floodplain soil

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Abstract

To study the interaction between chemical availability in a floodplain soil and physiological compartmentalization of internalised metals, bioaccumulation experiments were performed with the earthworm *Lumbricus rubellus*. Uptake and elimination kinetics of Zn and Cd were determined using radioisotopes, allowing for non-destructive measurements in time and allowing flux measurements of elements under homeostatic control. Two distinct compartments could be identified in the earthworm, with different affinities for Zn and Cd. The first compartment is thought to represent the pool of loosely-bound metals, whereas the second one represents a tightly bound storage fraction. A model based on this view provided an accurate description of the data set. Turnover rates were faster for Zn than for Cd. While the loosely-bound metal compartment determined Zn accumulation patterns, the magnitude of Cd accumulation was dominated by the behaviour of the storage compartment. Net accumulation over two weeks of Fe, Ni, Cu, Zn, Cd, Pb in the earthworms was at least two times higher than the absolute amount of metals present in the pore water of the soil, except for Ca. This supports the hypothesis that replenishment of the pore water concentration by desorption of metals from solid soil particles feeds metal uptake and that bioavailability cannot be seen as a static equilibrium. It is concluded that bioaccumulation estimates should be based on fluxes and account for the physiology of internalized metals. At least two internal and two external compartments are needed to describe metal kinetics in an accurate way.

4.1 Introduction

Earthworms are often used as test organisms to determine metal accumulation from soil (Van Gestel et al. 1993, Løkke and Van Gestel 1998, Spurgeon and Hopkin 1996, Osté et al. 2001). Most studies report relationships between internal and external concentrations (BSAF) where steady-state is assumed (Janssen et al. 1997, Lock and Janssen 2001). Surprisingly few studies have determined uptake and elimination kinetics as part of a dynamic process (Spurgeon and Hopkin 1999, Hendriks and Heikens 2000, Heikens et al. 2001). In laboratory accumulation studies, steady state is not always reached, especially not in earthworms, which tend to accumulate metals over a long period of time. Nevertheless, for interpreting test results and to allow for an extrapolation of laboratory data to the field situation, steady-state levels are needed (Peijnenburg et al. 1999).

Metal bioaccumulation kinetics is influenced by the physiology of the test organism. Following uptake, organisms are able to sequester internalized metals in various compartments (Rainbow 2002). Some sequestration forms have a high affinity for metals while others rapidly release metals. It is likely that the differences in metal sequestration have an impact on the ability of metal excretion by the animal. Morgan and Morgan (1990) found distinct differences in the distribution of various metals throughout the earthworms' body, whereby the sequestration on chloragocytes played a dominant role, resulting in different patterns of tissue accumulation (Morgan et al. 2002) and different tolerances (Morgan and Morgan 1998).

Our main aim was to obtain accurate Zn and Cd uptake and elimination rate constants for the earthworm *Lumbricus rubellus* exposed to floodplain soil, and approach internal compartmentalization. Isotopic labelling was used, which allows for the quantification of uptake and turnover kinetics in biota. The technique overcomes detection limitations, and allows insight into essential metal uptake even in the presence of highly-regulated body concentrations. For accurate accumulation kinetics, exposure concentrations in pore water and/or labile metal levels in the soil are restricted to be constant by replenishment. It was also investigated if pore water concentrations can explain the absolute amount of uptake by earthworms.

4.2 Materials and methods

Test organisms

Adult earthworms of the species *Lumbricus rubellus* were obtained from a commercial supplier (via CEH, Monks Woods, UK). Before the start of the experiments, organisms were kept for at least 3 weeks in a clay-rich soil with leaves of *Populus x canadensis* and stone fragments on top, under conditioned laboratory circumstances at 15°C with constant light. The same conditions were applied in the experiment.

Experimental design

A two-week uptake experiment was carried out, in which the earthworms were exposed to either radiolabelled soil (treatment A), or radiolabelled soil with unlabelled plant leaves on

top of it (treatment B). The latter treatment was applied to investigate the influence of food on the accumulation kinetics and on the health of the earthworms. Fresh weight (± st dev) of the earthworms was 710 ± 194 mg (n=6) in treatment A and 686 ± 73.7 mg (n=6) in treatment B. After 14 days, the organisms were transferred into unlabelled soil and internal concentrations were measured over a period of 32 days. For pragmatic and logistic reasons, uptake and elimination experiments were executed separately from each other. Three replicate treatments were performed. Earthworms were kept individually in jars, each jar containing 150 gram dry soil. Organisms kept in the same soil but not labelled with isotopes were occasionally monitored as a check for 'normal' behaviour and for background metal levels in their body.

Soil labelling

The soil used in the experiment was collected from the fresh water estuary floodplain "Ruitersplaat" in the Biesbosch, The Netherlands, having 17-19% organic matter, 22-28% clay, and pH (0.01 M $CaCl_2$) of 7.1-7.4. The soil was kept at a moisture content of 80% w/w (= 72% of maximum Water Holding Capacity). Soil was duo-labelled with ^{109}Cd and ^{65}Zn. Radioisotopes were certified and supplied as $^{109}CdCl_2$ (Amersham Biosciences, Buckinghamshire, UK) and $^{65}ZnCl_2$ solution (Perkin Elmer, Boston, USA). 1.25 MBq of both isotopes were added to 150 gram dry soil and 120 ml water. Specific activity of the isotopes was 18.5 MBq/ng for Cd and 12.3 MBq/µg for Zn. The isotopes added to soil were allowed to equilibrate for 14 days in the laboratory before starting exposure of the earthworms.

Measurements and analyses

Labile metal concentrations in soil were determined by using 0.01 M $CaCl_2$ extraction (Houba et al. 1996). Daily pore water was sampled using a permeable porewater micro probe (Rhizon SMS-MOM, The Netherlands), and total soil was sampled at intervals of two days.

The organisms were recollected daily and rinsed with tap water. ^{109}Cd and ^{65}Zn concentrations were determined using a Wallac gamma counter (Model 1480 3, EG&G company, Finland). To minimize stress, the animals were returned to the soil within 15 minutes. At the end of the experiment, unlabelled metal concentrations were determined in soil, food and animals. Earthworms were kept on wet filter paper for two days to void their gut prior to analyses. Two organisms were taken for measurement of total metal concentration after 14 d exposure, before transfer to unlabelled soil. Lyophilized organisms were individually digested in 7 ml 14.9 M HNO_3 (60% Suprapur, Merck) and 3 ml ultrapure water. The same procedure was used for 0.5 gram dried soil and 0.1 gram dried food. Certified reference material Dolt-2 (BCR, Brussels, Belgium), and blanks were treated in the same way as the samples. Samples were acid digested in a MULTIWAVE Microwave oven (Anton Paar GmbH, Austria). After digestion, the samples were analysed for metal concentrations using a Perkin-Elmer 4300 Dual View Inductively Coupled Plasma - Optical Emission Spectrometer (ICP-OES), equipped with a Perkin Elmer As-93Plus Autosampler (Perkin-Elmer, Shelton CT, USA). QA/QC measures included duplicate analyses; metal spikes were all within performance acceptance limits (90-110%), and so were the metal concentrations in the reference material Dolt-2.

Modelling

The internal concentrations measured in the uptake experiment (n=3 for each treatment) were taken together with the ones of the elimination experiment (n=3 for each treatment), which makes six individuals to model the uptake and elimination parameters. For each individual, 14 or 15 measurements in time were made. The internal concentrations in the organisms (Cw) were described by applying a two-compartment model (see Figure 1, and equations 1-4).

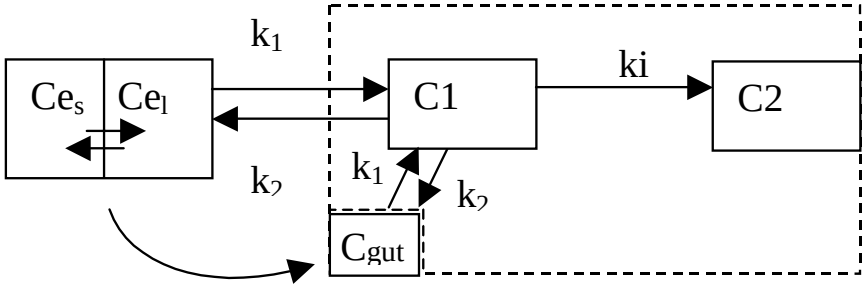

Figure 1: Scheme of uptake and elimination processes in the earthworm. The dashed block represents the animal, which is represented as a two-compartment accumulation model. Ce is the external concentration in soil that is divided in a metal pool bound to the solid phase (Ce_s) and in a soluble metal pool (Ce_l). Cgut is the concentration in the gut, which is not absorbed in the organism. The arrow k_1 represents the uptake rate constant, the arrow k_2 is the elimination rate constant and the arrow k_i is the transfer from the loosely-bound metal compartment (C1) towards the storage compartment (C2).

For the accumulation modelling, it was assumed that metal partitioning over the soil phases is faster than the rate of uptake by the animal, and therefore could be seen as a constant supply. In the accumulation phase, Cw = Cgut + C1 + C2, representing the concentration in the gut, a loosely-bound metal compartment (C1) and storage compartment for metals (C2), which is considered inert during the experiment. Cgut is the concentration in the worm, caused by metal present in the gut contents (MBq g^{-1} dry weight). Cgut was implemented in the two-compartment accumulation model as a model parameter, derived from the concentrations measured after 4 hours of exposure, which is the average time to fill the earthworm's gut (Jager et al. 2003). The increase of C1 and C2 with time, assuming constant Ce and a body burden of zero on t=0, can be described by:

$$C1(t) = \frac{k_1 \cdot Ce}{k_2 + k_i}\left[1 - e^{-(k_2 + k_i)t}\right] \qquad [1]$$

$$C2(t) = \frac{k_i \cdot k_1 \cdot Ce}{(k_2 + k_i)^2}\left[(k_2 + k_i)t + e^{-(k_2 + k_i)t} - 1\right] \qquad [2]$$

where C1(t) and C2(t) are the internal metal concentrations in compartments 1 and 2 (µg g^{-1} dry weight) at time t (d). Parameters to estimate are the uptake rate constant (k_1) expressed as gram soil per gram body weight per day ($g_{soil}\ g_{animal}^{-1}\ d^{-1}$), the elimination rate constant (k_2) and the rate constant (k_i) for metal transfer from the loosely-bound metal compartment towards the storage compartment, both expressed per day (d^{-1}).

After 14 days, the earthworms were transferred to soil without isotopic labels, and elimination kinetics were calculated using equations 3 and 4. Also here, Cw = C1 + C2

$$C1(t) = C1(14) \cdot e^{-(k_2+k_i)(t-14)} \quad [3]$$

$$C2(t) = \frac{k_i C1(14)}{(k_2 + k_i)}\left[1 - \exp^{-(k_2+k_i)(t-14)}\right] + C2(14) \quad [4]$$

where C1(14) and C2(14) are given by equations [1] and [2]. Uptake of radioisotopes excreted by the earthworms during the elimination experiment is considered to be negligible because of the use of a bulk of unlabelled soil.

Equations 1-4 were fitted simultaneously to the data for the six individual earthworms per treatment, programmed in Matlab 6.5 (The MathWorks Inc., Natick (MA), USA). To account for differences between individuals, k_1 was allowed to vary between individuals, but the other parameters were held the same for all earthworms. Confidence intervals were estimated using profile likelihood (see e.g. Meeker and Escobar 1995). Differences in parameter estimates between treatments were judged from the overlap of confidence intervals.

Replenishment of metals in the pore water by desorption from the labile and solid phases of the soil was calculated by comparing absolute uptake by the earthworms to the absolute amount of metals in the external pore water and in the 0.01 M $CaCl_2$ extractable metal pool of the soil. Calculations were extended with results derived from a comparable experiment with the same earthworm species and the same floodplain soil (M) as described in Vijver et al. (2003).

4.3 Results and discussion

Soil and pore water concentrations and labelled activities of Cd and Zn are given in Table 1. Total metal concentrations in soil and activity loads did not change during the exposure and are shown as average values.

The partitioning of non-labelled metals over solid and liquid soil phases, expressed as the partitioning coefficient Kd, was 4,166 ml g^{-1} for Cd and 12,537 ml g^{-1} for Zn. In the pore water only $5.13\ 10^{-4}$ µg ^{109}Cd L^{-1} and 0.62 µg ^{65}Zn L^{-1} was present as label, corresponding to less than 1% of total pore water concentration for Cd, and less than 3% for Zn. Disruption of metal speciation due to the addition of the labelled metals was therefore negligible.

All earthworms had healthy performance and no mortality occurred during the experimental period. No changes in mean fresh weight during the experiments and no differences between the treatments with and without food were found.

Table 1: Total (n=5), 0.01 M $CaCl_2$ extractable (n=2) and pore water (n=2) metal concentrations after equilibration and prior to the exposure of *Lumbricus rubellus*, and radiolabelled metal activities in pore water during exposure.

Medium, treatment	Cd (mg kg^{-1} d.w.)	^{109}Cd (MBq kg^{-1} w.w)	Zn (mg kg^{-1} d.w.)	^{65}Zn (MBq kg^{-1} w.w.)
Total concentration soil A	12.97	10.3 ± 0.13 *	2357	1.98 ± 0.50 **
Total concentration soil B	12.34	10.3 ± 0.15 *	2251	2.00 ± 0.50 **
	Cd (µg L^{-1})	^{109}Cd (kBq L^{-1} d.w.)	Zn (µg L^{-1})	^{65}Zn (kBq L^{-1}d.w.)
$CaCl_2$ soil A	3.84	15.14 ± 6.77	350	3.99 ± 1.30
$CaCl_2$ soil B	2.84	15.93 ± 8.24	201	4.24 ± 0.47
Pore water soil A	Cd (µg L^{-1})	^{109}Cd (kBq L^{-1})	Zn (µg L^{-1})	^{65}Zn (kBq L^{-1})
0 d	n.d.	9.5	n.d.	0.56
2 d	n.d.	7.5	n.d.	1.48
4 d	n.d.	7.0	n.d.	1.00
9 d	n.d.	6.5	n.d.	0.84
14 d	3.01	6.5	188	1.28
Pore water soil B	Cd (µg L^{-1})	^{109}Cd (kBq L^{-1})	Zn (µg L^{-1})	^{65}Zn (kBq L^{-1})
0 d	n.d.	9.5	n.d.	0.76
2 d	n.d.	7.0	n.d.	0.96
4 d	n.d.	8.0	n.d.	0.76
9 d	n.d.	6.5	n.d.	2.12
14 d	2.92	7.0	202	0.60

d.w. = dry weight basis, w.w. = wet weight basis; * corresponds to 18.54 MBq Cd kg^{-1} d.w., ** corresponds to 3.56 and 3.60 MBq Zn kg^{-1} d.w., n.d. = not determined. Pore water concentrations during the field sampling approx. 3.37 µg Cd L^{-1} (n=9) and 100.42 µg Zn L^{-1} (n=9). Treatment A is without food, treatment B is with unlabelled food addition.

Earthworms exposed to either ^{109}Cd labelled soil (n=6, treatment A) or ^{109}Cd labelled soil and unlabelled leaves (n=6, treatment B) did not reach steady-state by the end of the 14 day uptake phase. The accumulation pattern of earthworms exposed to soil without food (treatment A) is shown in Figure 2. Internal concentrations ranged between 2.25 and 3.50 MBq kg^{-1} fresh weight after 14 days. The compartment describing the stored metal fraction (C2) shows a nearly linear increase over the exposure period. After transfer of the earthworms to unlabelled soil, the first compartment (C1) emptied within several hours, after which the internal ^{109}Cd concentrations remained on the level reached in the storage compartment (C2).
^{65}Zn concentrations in earthworms exposed to either ^{65}Zn labelled soil (n=6) or ^{65}Zn labelled soil and unlabelled leaves (n=6) ranged between 0.32 - 0.56 MBq kg^{-1} fresh weight after 14 days. The accumulation pattern of earthworms exposed to the labelled soil without food can be seen in Figure 3. For Zn, the first compartment dominated the accumulation kinetics. After transfer of the earthworms to the unlabelled soil, the internal ^{65}Zn concentrations decreased within a couple of hours to reach a constant level, indicating a very fast turnover rate. Compared to Cd, for Zn the metal storage compartment (C2) was rather small.

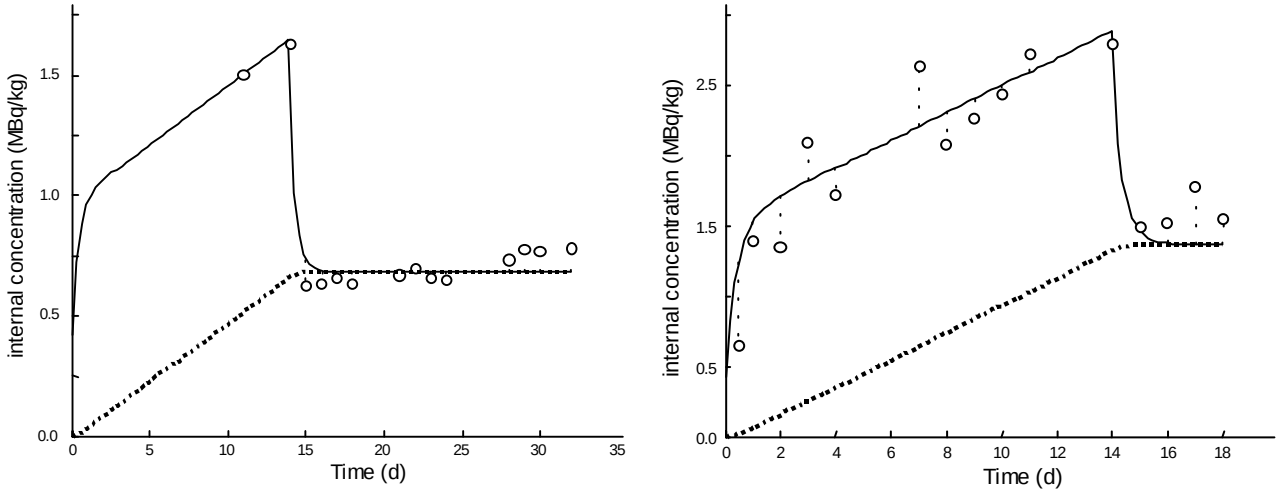

Figure 2: Accumulation of ^{109}Cd (in MBq kg^{-1} fresh weight) in *Lumbricus rubellus* exposed to ^{109}Cd labelled soil. Six individual animals were followed in time. As an example, one individual is depicted here during uptake and one during the elimination experiment. The solid lines show the accumulation patterns of the whole earthworm (Cw) and the dashed line the increase of the inert fraction (C2).

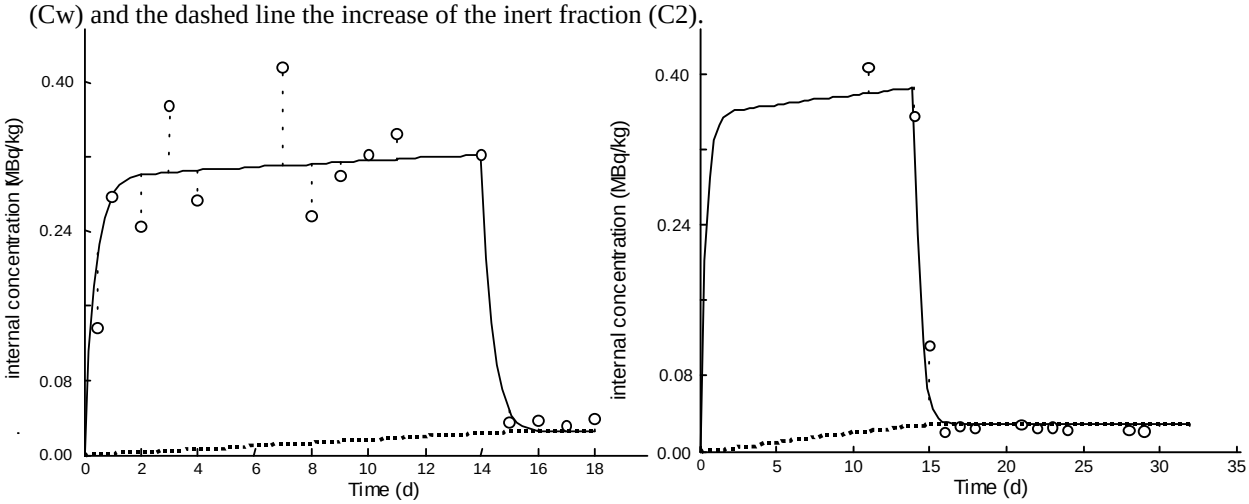

Figure 3: Accumulation of ^{65}Zn (in MBq kg^{-1} fresh weight) in *Lumbricus rubellus* exposed to ^{65}Zn labelled soil. Six individual animals were followed in time. As an example, one individual is depicted here during uptake and one during the elimination experiment. The solid lines are the accumulation patterns of the whole earthworm (Cw), the dashed line shows the increase of the inert fraction (C2).

Kinetic parameter values for Cd and Zn, calculated from the uptake and elimination patterns of *L. rubellus* exposed to labelled soil (treatment A) and to labelled soil with unlabelled food (treatment B), are given in Table 2.

Gut content after four hours of exposure was modelled to be 8.31 and 10.0 MBq kg^{-1} wet weight for ^{109}Cd, whereas for ^{65}Zn it was zero in both the treatments with and without leaves added on top of the soil.

The elimination rate constant (k_2) from the loosely-bound metal pool (C1) was high for both Cd and Zn, ranging between 2.30 and 3.53 d^{-1}. Addition of unlabelled food slightly stimulated excretion from the loosely-bound fraction for both metals, although not significantly. The ki reflecting the rate constant for metal transfer from C1 towards the C2 (metal storage compartment) was a factor of 10 higher for Cd than for Zn. C1 has more affinity for Zn than for Cd. This was expected, since Zn is an essential element and required for many metabolic processes and therefore likely to show high turnover rates in C1. Moreover, the low Zn

concentrations (see Figure 3) mean that hardly any Zn was above the requirements of C1. Cd was mainly transported to storage sites with high affinities for metals (C2), in that way reducing its toxicological effects.

Table 2: Estimates for kinetic parameters (see also Figure 1) for ^{109}Cd and ^{65}Zn accumulation in six individual earthworms, *Lumbricus rubellus*, exposed to radiolabelled soil (left column) and to radiolabelled soil and unlabelled food (right column). k_1 (g_{soil} g_{animal}^{-1} d^{-1}) is the uptake rate constant either from soil without food exposure ($k_{1'soil}$), or from labelled soil and unlabelled food (k_{1soil}). The uptake rate constants are individual-specific; other parameters modelled are species and treatment-specific. k_2 (d^{-1}) is the elimination rate constant from the loosely-bound metal compartment (C1), ki is the rate constant describing metal transfer from the loosely-bound metal compartment towards the storage compartment (C2), and Cgut the concentration in worm due to content of the gut (MBq kg^{-1}). Minimum and maximum value are likelihood-based 95% confidence intervals.

	value	min-max		value	min-max
^{109}Cd labelled soil			^{109}Cd labelled soil with unlabelled food		
k2	2.30	1.34 - 4.64	k2	3.10	1.67 - 3.10
C gut	8.31	0 - 14.8	C gut	10.0	0 - 14.8
ki	0.09	0.05 - 0.16	ki	0.08	0.06 - 0.10
k1' soil	0.335		k1 soil	0.443	
k1' soil	0.268		k1 soil	0.260	
k1' soil	0.242		k1 soil	0.279	
k1' soil	0.196		k1 soil	0.342	
k1' soil	0.134		k1 soil	0.284	
k1' soil	0.155		k1 soil	0.310	
	value	min-max		value	min-max
^{65}Zn labelled soil			^{65}Zn labelled soil with unlabelled food		
k2	2.57	1.37 - 3.71	k2	3.53	2.39 - 9.04
C gut	0	0 - 0.46	C gut	0.00	0 - 0.46
ki	0.006	0.004 - 0.02	ki	0.008	0.006 - 0.009
k1' soil	0.584		k1 soil	0.821	
k1' soil	0.388		k1 soil	0.631	
k1' soil	0.400		k1 soil	0.560	
k1' soil	0.390		k1 soil	0.693	
k1' soil	0.295		k1 soil	0.567	
k1' soil	0.464		k1 soil	0.587	

The observed differences in metal uptake, such as a fast uptake of essential metals reaching equilibrium within a few days, and non-equilibrium levels for non-essential metals, are in agreement with the findings of Spurgeon and Hopkin (1999). However, quantitative comparison of the elimination rate constants to literature data is difficult. All studies have as starting point that when an animal is exposed to contaminated soil, the internal concentration will rise until equilibrium is reached between uptake and elimination. Following transfer to clean soil, the internal concentration will decrease until the animal is clean again. Nevertheless, elimination curves show obvious plateaus that even after 100 days of elimination indicate that the earthworms still contain a residual amount of unexcreted metal (e.g. Spurgeon and Hopkin 1999, Sheppard et al. 1997). In these cases, the elimination curves are derived using a one-compartment model that combine the two compartments C1 and C2. Our results show that the earthworm *Lumbricus rubellus* is able to a certain extent to eliminate Cd and Zn from the loosely-bound metal compartment C1. When C1 is empty, further elimination seems impossible. Considering only the elimination rate constant for Cd

from C1, a k_2 value of approximately 3 d^{-1} is relatively high compared to the Cd elimination rate kinetics reported for oligochaete species, which range between 0.001 and 0.1 d^{-1} (Hendriks and Heikens 2001) and for Zn of 1.5 d^{-1} (Spurgeon and Hopkin 1999). Indications that Cu and Pb excretion is a biphasic process, distinguished by fast elimination kinetics followed by slow release ascribable to the " inert" stored metals was also found by Marinussen et al. (1997). However, as stated before, in all other studies elimination rate constants represent the average of very fast elimination from C1 together with an inert storage (C2). The results, however, show that the overall accumulation (Cw) of Cd is almost entirely determined by the storage compartment (C2). Zn accumulation is mainly described by the loosely-bound fraction (C1), but also for this element the inert compartment ensures that elimination to a tissue concentration value of zero is never reached.

Uptake rate constants derived from soil exposure without food and from exposure to labelled soil with unlabelled leaves did not differ significantly. Obviously, the addition of food did not affect metal uptake from soil. This observation was expected because available evidence suggests that uptake of metals by earthworms occurs primarily from the soil rather than from food, although *Lumbricus rubellus* is an epigeic earthworm species feeding on litter (Spurgeon and Hopkin 1999). Comparable experiments performed with the litter-feeding isopod *Porcellio scaber*, however, showed predominant uptake via the food (Vijver et al. Chapter 5). The difference can be ascribed to the physiological differences in exposure routes between the soil-dwelling isopod and the earthworm that is completely surrounded by pore water and solid soil particles.
Summarizing, the results show that the elimination of Zn and Cd are likely to be based on the same principles. Also the uptake rates of Zn and Cd did not differ more than a factor of two. The redistribution rate (ki), however, is much larger for Cd than for Zn, and the organisms seem to be able to discriminate between the metals in this respect.

The underlying assumption in the modelling was that the external concentration is constant. According to the pore water hypothesis (e.g. Van Gestel 1997), soluble metals are the predominant contributors for uptake by earthworms (Vijver et al. 2003). To maintain a constant exposure level, desorption must take place to compensate for metal uptake by the earthworms. In the experiment described in this paper, two mechanisms contribute to metal uptake by earthworms via the pore water. The first mechanism is uptake of water containing metals in the soluble phase. Water loss in earthworms in soil is estimated to amount 10-20% of the body weight per day (Lee 1985), which must be compensated for by water uptake across the epidermis and from the gut content. For earthworms with a fresh weight of 500 to 700 mg, this means that 0.05 to 0.14 ml water per day should be replenished. Porewater concentrations of Cd and Zn were approximately 3 and 200 µg L^{-1} respectively (Table 1). Within 14 days of exposure, the water uptake, therefore, corresponds to an average uptake of 0.0042 µg Cd and 2.6 µg Zn per earthworm. This is negligible compared to the total amount of metals taken up by the earthworms in this experiment.

The second mechanism is diffusion of metals from the pore water into the earthworm. In earlier research (Vijver et al. 2003), by blocking the mouth of earthworms, metal uptake by *Lumbricus rubellus* was separated into a dermal route and a gut route. Cu and Pb accumulation appeared to be fully determined by the dermal route, whereas ingestion contributed for 0-17% and 21-30% respectively to internal Cd and Zn concentrations. Table 3 shows the calculation of the amount of metals that should be replenished from the solid phase towards the soluble phase to describe metal accumulation in the earthworm *Lumbricus rubellus*. The absolute amount of metal taken up by the earthworms is multiplied by the total biomass of the earthworms exposed, corrected for the percentage taken up via the dermal route. The metal concentration in the pore water is multiplied by the amount of total pore water. Release of the metals adsorbed to the solid phases of the soil to the pore water is the so-called replenishment factor. Calculations were done on a range of metals analysed using ICP-equipment. The relative importance of the dermal uptake route for Ca, Fe, and Ni was derived as described by Vijver et al. (2003).

Table 3: Replenishment factors indicating the times pore water metal concentrations have to be renewed by desorption to explain the measured metal concentrations in *Lumbricus rubellus*. From the metal concentrations in earthworms (Cw) at t=0 and t=14 and the body weight, the absolute increase of the body burden is estimated. The percentage of dermal uptake was estimated from experiments in which worms were orally sealed (Vijver et al. 2003). Concentrations in pore water were Ca 290.8 mg L^{-1}; rest of metals in µg L^{-1}, Fe 15.24, Ni 6.20, Cu 42.78, Zn 194.7, Cd 2.97, Pb 1.00, the vessel contained 268 ml pore water, and total biomass of earthworms (n=4) was 316 gram d.w.

	Cw (µg/g)		absolute metal amount	absolute metal amount	Dermal uptake	Replenishment
	t(0)	t(14)	in d.w. worms (µg)	in pore water (µg)	%	factor
Ca	3321	9413	577	77943	87	0.02
Fe	1363	5659	449	4.09	96	245
Ni	0.58	15.5	1.36	1.66	84	1.83
Cu	8.79	92.3	9.09	11.46	100	1.77
Zn	457	1190	55.8	52.2	70	2.39
Cd	5.59	13.5	0.72	0.80	83	2.02
Pb	22.6	95.2	7.89	0.27	100	66.0

t(0) = initial concentration, t(14) is concentration after 14 d of exposure

As can be seen in Table 3, for most elements pore water concentration contained less than the total amount accumulated by the earthworms. Therefore, pore water concentrations need to be restored by the metals desorbing from the solid phases of the soil. The metals Ni, Cu, Zn, and Cd all are taken up by the earthworms in amounts being approximately a factor of 2 higher than directly available in the pore water. The volume of the pore water that is in contact with the animal is relatively small, and calculation of metal depletion was based on the total amount of pore water in the test vessel. Therefore, the replenishment factor is under-estimated, and metal desorption on a local scale will probably be higher. For Fe and Pb, a large replenishment factor was calculated, probably because their affinity for earthworm tissue is higher than their sorption to clayish soils. The amounts of Ca in pore water are much higher than the total amount taken up by the earthworms. As a consequence no Ca desorption from the solid phases is necessary to explain Ca uptake.

For most metals, biological uptake kinetics were shown to be faster than the chemical release kinetics in sediments (at WHC >100%) (Vink 2002). Using implanted rhizons with a length of 10 cm in our experiment, the pore water concentrations measured daily were shown to

remain constant (for concentrations of ^{109}Cd and ^{65}Zn, see Table 1). It may, therefore, be concluded that the release in this floodplain soil (at WHC 72%) matches the uptake by worms.

Since desorption of metals from the soil solid phase also contributes to metal uptake by earthworms, this explains the empirical finding that metal uptake in earthworms is often better correlated to a 0.01 M $CaCl_2$ extraction than to pore water metal concentrations (Posthuma et al. 1998). As shown in Table 1, the $CaCl_2$ extractable activity loads were twice as high as the pore water loads. Therefore, these extractable fractions seem better predictors of the total amount of radiolabels taken up by the earthworms. There are a numerous different extraction methods, such as 0.01 M HCl extraction (Impellitteri et al. 2003) and 0.43 M HNO_3 solution (Beckett 1989), reflecting the labile metal pool of the soil. These methods are used to assess chemical availability simulating bioaccumulation (Houba et al. 1996). Another method to predict potentially bioavailable fractions is the use of isotopic exchange data, which can be applied to determine the soil buffering capacity and the depletion of isotopes from soil solution (Hamon et al. 2002). Assuming that soil and pore water have a different ratio of labelled metals and non-labelled metals, that can be compared to the differences between the isotopic composition in organisms, this can provide information on the pore water metal pool as being the source of uptake for the studied animal (Smolders et al. 1999). Calculating the isotopic composition for both Cd and Zn in *L. rubellus* and comparing it to the ratio of labelled and unlabelled metals in the pore water of the floodplain soil, shows that uptake cannot totally be described by the pore water concentrations. This agrees with the replenishment factors for Cd and Zn given in Table 3. However, all these soil extraction methods and isotopic composition calculations have the disadvantage that they have a static character, and refer to the average accumulation of an organisms over time, rather than estimating the external metal pool from which uptake occurs. Moreover, they neglect the effect of replenishment from various loosely-bound metal fractions towards metal fractions that can easily be taken up by biota.

The results make clear that bioavailability cannot be dealt with as a static equilibrium. For the prediction of bioavailability, replenishment of metals from the solid phases to the soluble phases in the soil should be involved and cannot be seen as a constant infinitive metal supply. More research is needed on the uptake kinetics of metals from soil phases. It is likely that the modelling should be extended by some external compartments describing the chemical release kinetics of metals bound to solids towards metals in the pore water.
In bioaccumulation studies, the internal metal distribution should be taken into account to explain different accumulation patterns. Metals can be retrieved from a loosely-bound compartment or can be found in a storage compartment from which elimination is negligible. Many studies describe elimination rate constants based on one-compartment modelling, however, a smooth elimination curve to zero is hardly ever seen for earthworms. Two-compartment accumulation modelling, accounting for a loosely-bound and an inert storage for metals, is required to describe the typical Zn and Cd accumulation kinetics in earthworms.

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Chapter 5

Kinetics of Zn and Cd accumulation in the isopod *Porcellio scaber* exposed to contaminated soil and/or food

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Submitted

Chapter 5
Kinetics of Zn and Cd accumulation in the isopod *Porcellio scaber* exposed to contaminated soil and/or food

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Abstract

To derive accumulation kinetics from different exposure matrices and account for the contribution of different exposure routes, the isopod *Porcellio scaber* was exposed to either soil or food, and a combination of both. Accurate uptake and elimination kinetics of Zn and Cd were determined using radioisotopes, allowing non-destructive time measurements. Modelling was done using first order kinetics, accounting for internal distribution of metals over an exchangable and a storage compartment. Elimination kinetics, together with the metal fraction that is compartmentalized in a storage fraction, were found to be predominantly responsible for the Cd steady-state level reached in the isopods. Zn turnover kinetics were relatively fast within the first days of exposure and elimination. This was followed by slow Zn kinetics from the storage compartment. The storage fraction appeared to be inert for elimination of both Zn and Cd and the percentage metals stored was influenced by the uptake source.

The quantity of metals taken up from soil or food depends on the concentration. For both Zn and Cd, the uptake rate constant from soil equalled the uptake rate constant from food, moreover, the uptakes from the two routes were shown to be additive. Therefore, it is concluded that the relative contribution of each route (via soil or food) is driven by the partitioning of metals between soil and food.

5.1 Introduction

In a metal-contaminated ecosystem, soil-dwelling organisms are not only exposed to metals in the soil, also the litter layer in which metals are most effectively accumulated (Martin et al. 1982) adds to the risk. This above average exposure to metals holds especially for detritivorous organisms, such as isopods, because their food (organic matter) and their microhabitat coincide with the places – upper layer of the soil – where metal accumulation takes place. Metal accumulation in terrestrial isopods has been studied intensively (Dallinger and Prosi 1988, Witzel 1998, Hopkin and Martin 1982) and these organisms are recognized as suitable bioindicators of metal pollution (Paoletti and Hassall 1999). Their ability to accumulate high metal concentrations can be ascribed to an efficient storage organ, the hepatopancreas, which contains up to 90% of the metals in the isopod's body (Hopkin 1990). The internal sequestration in metabolically active metal pools and storage compartments is likely to have an impact on elimination abilities of the animal. These aspects are hardly accounted for in bioaccumulation studies, although there is a strong ecophysiological evidence for this (Rainbow 2002).

Metals may be taken up via different routes and traffic through the body until the targets are reached. Metals taken up orally enter the digestive tract from where they go directly from the gut fluid or via the typhlosole channels to the hepatopancreas (Hames and Hopkin 1991a). They may also diffuse into the haemolymph, from where they are partly excreted via the kidneys (Donker et al. 1996). Another route of metal uptake can be via the pleopods under the abdomen of the isopod, which absorb water from the environment by capillary action (Sutton 1972). Metals taken up via this route will circulate in the haemolymph through the entire body until a target organ is reached.

Although metal accumulation is the net result of uptake from different routes of exposure, accumulation kinetics usually are derived under laboratory settings in which organisms are exposed to either contaminated soil or food. In that case, accumulation kinetics are relatively easy to describe as only one single source is considered. Single exposure, however, rarely occurs in polluted ecosystems, as elevated levels occur in both soil and litter layers.

In this study, different exposure regimes were used, focusing on either soil or food exposure or a combination. The main aim was to investigate the relative contribution of each uptake route for Zn and Cd accumulation and quantify the impact of the effective storage capacity on metal kinetics in the isopod *Porcellio scaber*. It was hypothesized that metal uptake from food predominates over uptake from soil and pore water.

Kinetics were determined for the non-essential metal Cd and the essential metal Zn by using radioisotopes. Radioisotopes with an atomic mass greater than 35 behave in biological tissues like stabile metals and animals do not discriminate between the isotopes (e.g. Croal et al. 2004). The radioisotope-technique is especially useful to provide insight in the turnover and specific accumulation kinetics of essential metals that often occur at highly regulated concentrations in animals. Furthermore, the technique allowes measurement of individual organisms over time.

5.2 Materials and methods

Test animals

Adult isopods *Porcellio scaber* Latreille, 1804, were collected from an uncontaminated garden on sandy soil in Bilthoven, The Netherlands. Before the start of the experiment, isopods were kept in a clay-rich soil with poplar leaves *Populus x canadensis* on top, under constant laboratory conditions for at least three weeks at 15 °C with constant light. Shelter for the isopods was provided by placing some stone fragments on the soil surface. The same conditions were applied during the experiments.

Experimental design

A two-week uptake experiment was carried out, in which the test animals were exposed to soil or food labelled with radioisotopes. After 14 days, isopods were transferred to unlabelled soil or food and the decrease of internal concentrations was determined up to 32 days. To quantify the impact of more than one uptake route on metal accumulation by soil organisms, different exposure regimes were applied. The experimental set up is given in Table 1.

Table 1: The experimental treatments applied to determine the uptake and elimination kinetics of radiolabelled Zn and Cd in the isopod *Porcellio scaber*.

treatment	soil	food
A	labelled	no
B	labelled	unlabelled
C	labelled	labelled
D	unlabelled	labelled

All experimental treatments were performed in triplicate. In each test jar one isopod was placed. For pragmatic and logistic reasons, uptake and elimination experiments had to be executed separately. Isopods kept in the same soil although unlabelled and with unlabelled food were occasionally monitored as a check for ‘normal’ behaviour and for the analysis of background metal levels in the isopod’s body.

Soil and food labelling

The soil used in the experiment was collected from the fresh water estuary floodplain “Ruitersplaat” in the Biesbosch, The Netherlands, having 17-19% organic matter, 22-28% clay, and a pH (0.01 M $CaCl_2$) of 7.1-7.4. The soil was kept at a moisture content of 80% w/w (= 72% of maximum Water Holding Capacity). Food was supplied *ad libitum* to the isopods in pieces sized 4-8 mm on stone fragments placed on the soil surface. The food consisted of soaked poplar leaves *Populus x canadensis*. Soil and food were labelled with radioisotopes as duo-label with ^{109}Cd and ^{65}Zn. Radioisotopes were certified and supplied as $^{109}CdCl_2$ (Amersham Biosciences, Buckinghamshire, UK) and $^{65}ZnCl_2$ solution (Perkin Elmer, Boston, USA). Specific activity of the isotopes was 18.5 MBq/ng for Cd and 12.3 MBq/µg for Zn. Of both isotopes, 1.25 MBq was added to 150 gram dry soil and 120 ml water. Food of 0.06 gram fresh weight was soaked in a 5 ml solution containing 1.25 MBq of both isotopes. The

isotopes added to soil and food were allowed to equilibrate for 14 days in the laboratory before starting exposure of the isopods.

Measurements and analyses

Daily, the isopods were recollected from the jars, rinsed with tap water, and ^{109}Cd and ^{65}Zn concentrations were determined using a Wallac gamma counter (Model 1480 3, EG&G company, Finland) while the animal was held in a measuring vessel. To avoid stress, these measurements were performed within 15 minutes. Soil and food were frequently sampled and measured on the gamma counter. At the end of the experiment, non-radioactive metal concentrations were determined in soil, food and animals. Isopods were allowed to void their gut for one day (Donker et al. 1996, Hartenstein 1964) and subsequently used for analyses. Four organisms were taken for measurement of total metal concentrations after 14 d exposure, before transfer to unlabelled soil and food. Total metal analyses were performed after freeze-drying the individual animals, and digestion in 7 ml 14.9 M HNO_3 (60% Suprapur, Merck) and 3 ml ultrapure water. The same procedure was used for 0.5 gram dried soil and 0.1 gram dried food. Metal analyses were done using a Perkin-Elmer 4300 Dual View Inductively Coupled Plasma - Optical Emission Spectrometer (ICP-OES), equipped with a Perkin Elmer As-93Plus Autosampler (Perkin-Elmer, Shelton CT, USA). QA/QC measures included duplicate analyses, metal spikes were all within performance acceptance limits (90-110%), and so were the metal concentrations in certified reference material Dolt-2 (BCR, Brussels, Belgium).

Modelling

The internal metal concentrations measured in the accumulation experiment (n=3 for each treatment) were taken together with those from the elimination experiment (n=3 for each treatment) to model uptake and elimination parameters. The increase of internal concentrations in the isopods (Ci) during 14 days of exposure to soil or food was estimated from uptake kinetics by applying a one-compartment model:

$$Ci(t) = Cgut + \frac{a}{k_2}\left[1 - e^{-k_2 t}\right] \qquad [1]$$

where Ci(t) is metal concentration in the isopod (MBq g^{-1} dry weight) at time t (d); Cgut is the concentration in the animal caused by gut contents (MBq g^{-1} dry weight). k_2 is the elimination rate constant (d^{-1}), and a is the uptake flux (MBq food or soil per g animal per day, on a dry weight basis), which equals $k_1 \cdot Ce_{food\ or\ soil}$, in which k_1 is the uptake rate constant ($g_{soil}\ g_{animal}^{-1}\ d^{-1}$) and Ce is the external concentration in soil and/or food.

After 14 days, the animals were transferred to soil and food without isotopic labels, and elimination kinetics were described using equation 2. This equation was extended with an inert fraction that accounts for metal translocation inside the isopod's body towards storage pools from which no elimination occurs.

$$Ci(t) = Ci(14)\left[Fi + (1 - Fi)e^{-k_2(t-14)}\right] \quad [2]$$

where Ci(14) was calculated from equation 1. Fi is the fraction (ranging from 0 - 1) that cannot be eliminated and is stored in the body. Cgut is excluded from equation 2, because this compartment is assumed to be rapidly refreshed and thus does no longer contain radioisotopes at the first measurement after 4 hours. Uptake of radioisotopes excreted by the isopods during the elimination experiment is considered to be negligible because of the large amount of unlabelled soil applied.

Equations 1 and 2 were simultaneously fitted to the data for six individual organisms per treatment, programmed in Matlab 6.5 (The MathWorks Inc., Natick (MA), USA). To account for inter-individual differences in accumulation, it turned out to be sufficient to allow the uptake flux (a) to differ between animals. This was motivated by experimental observations showing that feeding rates are the largest source of variability in experiments with isopods (Donker et al. 1993). For the other parameters (Fi, k_2, and Cgut) a single value was estimated over all six replicates. Confidence intervals were generated using the profile likelihood (e.g. Meeker and Escobar 1995). Differences in the kinetic parameters between treatments were determined by checking overlap of confidence intervals.

From treatment A, in which no food was added, $k_1'_{soil}$ was derived, which is the uptake rate constant for soil exposure only. From treatment B, k_{1soil} was derived under circumstances where unlabelled food was available. From treatment D, k_{1food} was derived under circumstances that unlabelled soil was available. The uptake in isopods exposed in treatment C is a combination of uptake from soil and food. Assuming that the two exposure pathways can be added, the total flux into the body can be written as:

$$a_{combination} = a_{soil} + a_{food} = k_{1\,soil} C_{soil} + k_{1\,food} C_{food} \quad [3]$$

and the predicted uptake from experiment B and D is compared to the measured uptake.

5.3 Results

Soil and food concentrations and labelled activities of Cd and Zn are given in Table 2. The soil to which the isopods were transferred to determine the elimination of the radiolabelled metals contained 34.1 mg Cd kg^{-1} and 632 mg Zn kg^{-1}, for Cd this concentration is higher than in the uptake soil. Label activity, however, was below detection limit in the elimination soils and labels were used to determining uptake and elimination kinetics.

Table 2: Total metal concentrations (n=3) and radiolabelled metal activities in soil and food during the uptake experiment with *Porcellio scaber*. A, B, C, D are the treatments described in Table 1, treatment A does not contain food.

Medium, treatment	Cd (mg kg^{-1} d.w.)	^{109}Cd (MBq kg^{-1} w.w)	Zn (mg kg^{-1} d.w.)	^{65}Zn (MBq kg^{-1} w.w.)
Soil A	13.0 ± 0.40	10.3 ± 0.13	2357 ± 55.9	1.98 ± 0.46
Soil B	12.3 ± 1.05	10.3 ± 0.15	2251 ± 133	2.00 ± 0.49
Soil C	12.6 ± 0.54	10.3 ± 0.50	2223 ± 66.5	1.98 ± 0.83
Soil D	11.3 ± 1.91	0.11 ± 0.09	2281 ± 174.6	0.24 ± 0.22
Food B	4.10 ± 1.79	1.75 ± 1.75	226 ± 109	0.65 ± 0.48
Food C	9.04 ± 2.58	3,460 ± 2,289	606 ± 303	391 ± 209
Food D	9.51 ± 5.86	3,438 ± 1,937	636 ± 334	388 ± 181

d.w. = dry weight, w.w. = wet weight. Label concentration in soil can be calculated on basis of d.w. by taking the water content of 80% into account.

No mortality of isopods during the experimental period occurred, and no loss of fresh weight of the isopods (61.8 ± 12.8 mg) was recorded, indicating healthy animals. In treatment D, one individual behaved in a deviant way and only two individual isopods could be monitored over the entire experimental duration.

^{109}Cd kinetics in isopods

^{109}Cd concentrations in *Porcellio scaber* increased with exposure time to labelled food, soil or the combination. Isopods exposed to ^{109}Cd labelled soil (treatment A and B) did not reach a steady-state concentration during 14 days of exposure, neither did the isopods exposed to labelled food (treatment D). The magnitude of body residues after 14 d were 3.5, 3.0 and 300 MBq kg^{-1} fresh weight in the exposure treatments A, B and D respectively. As an example, Figure 1 depicts the development of ^{109}Cd concentration in one individual exposed to treatment B. Animals exposed to both labelled soil and food (treatment C) reached steady-state after 10 to 12 days. The body residues from soil and food for the isopods reached a maximum of approximately 375 MBq kg^{-1} fresh weight (see Figure 2). Differences in the magnitude of accumulation between individuals are caused by differences in the label concentrations, which were higher in food than in soil.

After transfer to non-labelled soil and food, the internal ^{109}Cd concentration of isopods exposed via soil decreased (treatments A and B). Internal concentrations of animals exposed via food only slowly decreased after the transfer to unlabelled food and soil. The rapid decrease of the concentration within the first four hours is ascribed to refreshment of the gut content, in this time animals filled their gut with unlabelled material. The elimination pattern after these initial hours is explained by the elimination rate.

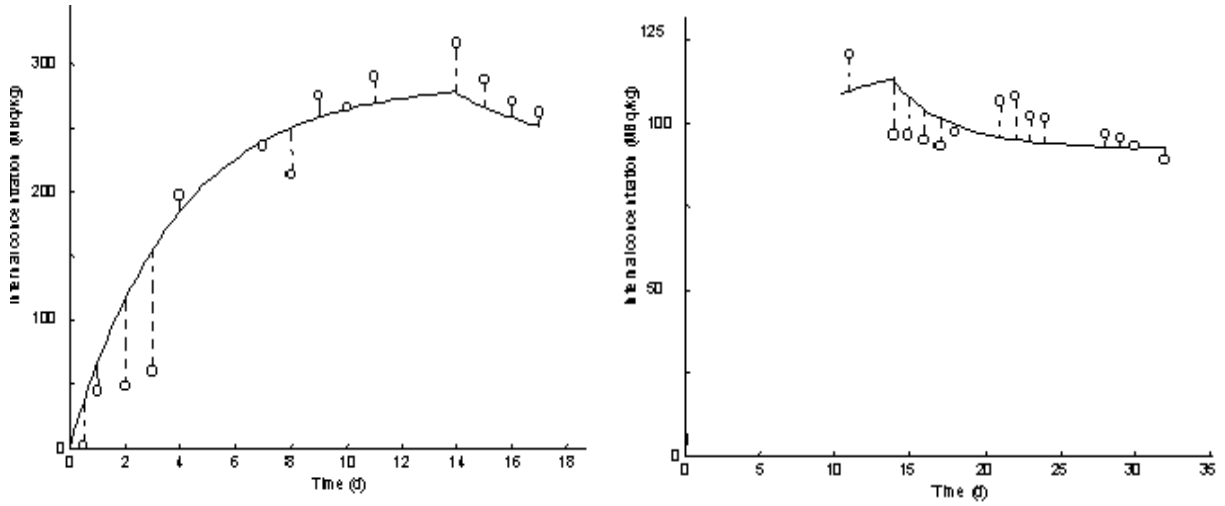

Figure 1: Accumulation of ^{109}Cd (in MBq kg^{-1} fresh weight) in *Porcellio scaber* exposed to ^{109}Cd labelled soil and unlabelled food (treatment B). Six individual animals were followed in time. As an example, one individual is depicted here during uptake and one during the elimination experiment.

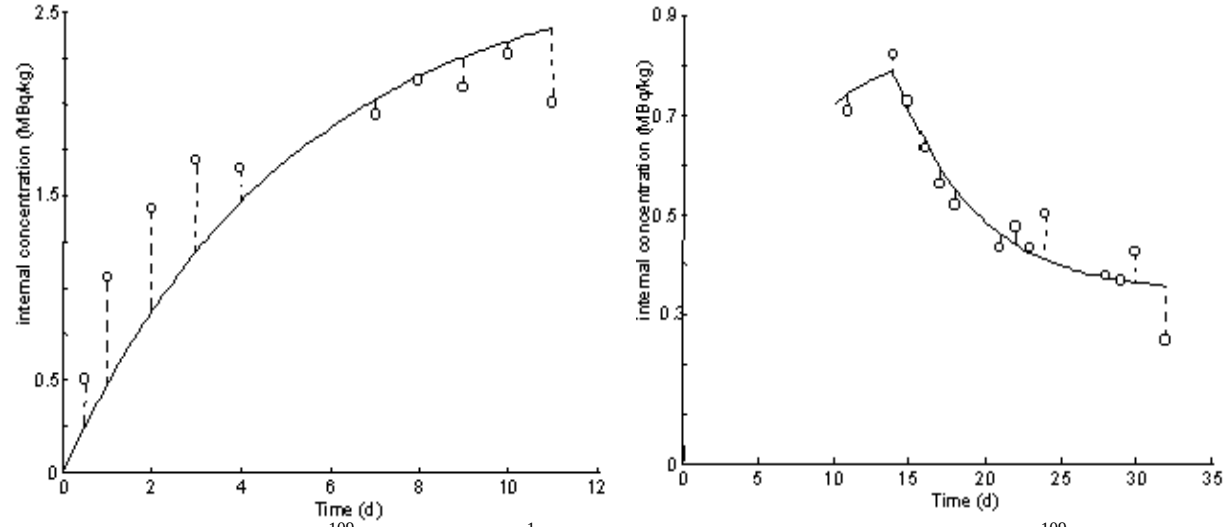

Figure 2: Accumulation of ^{109}Cd (in MBq kg^{-1} fresh weight) in *Porcellio scaber* exposed to ^{109}Cd labelled soil and ^{109}Cd labelled food (treatment C). Six individual animals were followed in time. As an example, one individual is depicted here during uptake and one during the elimination experiment.

Non-radioactive Cd levels in sacrificed animals after 14 days exposure amounted 8.24 ± 1.74 (n=4). The animals at the end of the experiments, so after 32 days in treatments A, B, C, and D reached 6.70 ± 1.92 (n=3), 5.83 ± 1.86 (n=3), 5.77 ± 0.45 (n=3) and 3.75 ± 0.38 (n=2) mg Cd kg^{-1} dry weight, respectively. As can be seen when comparing Figures 1 and 2, ^{109}Cd label levels differed approximately a factor of 100 after 32 days and non-radioactive Cd concentrations were similar, reflecting the high specific activity of the ^{109}Cd label used.

^{109}Cd levels depicted in Figures 1 and 2 are dependent on the doses of the radioactive ^{109}Cd label added to the external matrix and, therefore, do not reflect the difference in uptake route. Accumulation kinetics do, however, provide insight in the concentration fluxes taken up via each source. Kinetic parameter values for Cd calculated from the uptake and elimination patterns are given in Table 3.

Table 3: Estimates of kinetic parameters for ^{109}Cd accumulation in six individual isopods, *Porcellio scaber*, exposed to the different treatments described in Table 1. Uptake rate constants (k_1) from either food or soil were estimated for each individual separately, while the other parameters were assumed to hold for all animals. Uptake rate constants were derived from the uptake fluxes (a) by taking the external concentration into account. k_1 (g_{soil} g_{animal}^{-1} d^{-1}) is the uptake rate constant for an individual animal either from soil in the absence of food ($k_{1'soil}$), from labelled soil in the presence of unlabelled food (k_{1soil}), or labelled food in the presence of unlabelled soil (k_{1food}), k_2 (d^{-1}) is the elimination rate constant, Fi is the fraction stored in inert form and C gut the content of the gut (MBq kg^{-1}). Minimum and maximum value are likelihood-based 95% confidence intervals.

	value	min-max		value	min-max
treatment A			treatment B		
k_2	0.085	0.04 - 0.13	k_2	0.191	0.13 - 0.35
Cgut	0.285	0.152 - 0.403	Cgut	0.00	0.00 - 0.084
Fi	0.36	0.05 - 0.48	Fi	0.43	0.34 - 0.53
$k_{1' soil}$	0.018		$k_{1 soil}$	0.048	
$k_{1' soil}$	0.027		$k_{1 soil}$	0.051	
$k_{1' soil}$	0.038		$k_{1 soil}$	0.056	
$k_{1' soil}$	0.017		$k_{1 soil}$	0.016	
$k_{1' soil}$	0.009		$k_{1 soil}$	0.021	
$k_{1' soil}$	0.024		$k_{1 soil}$	0.035	
treatment C			treatment D		
k_2	0.258	0.16 - 0.41	k_2	0.177	0.06 - 0.36
Cgut	1.24	0.00 - 2.82	Cgut	0.00	0.00 - 11.4
Fi	0.83	0.8 - 0.9	Fi	0.64	0.0 - 0.64
			$k_{1 food}$	0.020	
			$k_{1 food}$	0.008	

Cd concentration of the gut (C gut) calculated after four hours of exposure, was rather high in the treatment with labelled soil and no food (A). Gut content was zero in the treatment with labelled soil and unlabelled food (B). This indicates that the isopod prefers to feed on poplar leaves instead of soil. Isopods in the treatment with labelled soil and labelled food (C) had a high gut content after four hours, again ascribed to food reflecting the gut content. Gut content was almost zero with a high uncertainty in isopods exposed to unlabelled soil and labelled food (D). In this treatment only two isopods were monitored and the uncertainty may thus be explained by the fact that one isopod directly started feeding while the other did not.

The elimination rate constant (k_2) was similar for all different treatments, except for treatment A in which no food was added.

The inert fraction reflects the metal fraction in which Cd is captured with high affinity, resulting in slow or negligible elimination from this storage compartment. In the animals exposed to labelled soil, the storage fraction reached an average of 36 and 43%, in treatments A and B respectively. Animals exposed to labelled food had an inert fraction of 83 and 64%, in treatments C and D respectively. Uptake via food thus shows higher inert storage of Cd. In general, uptake flux via both routes (soil and food) is rapid and the animal is translocating excess Cd as fast as possible towards storage (Fi) and excretion (see high k_2 value).

The mean (± standard deviation) uptake rate constants (k_1) for treatments A, B and D did not significantly differ from each other and were 0.022 ± 0.010, 0.038 ± 0.017, 0.014 ± 0.008 g_{soil}/g_{animal} per day, respectively.

^{65}Zn kinetics in isopods

^{65}Zn concentrations in *Porcellio scaber* increased with time during exposure to labelled food, soil or the combination. Within 14 days of exposure, steady-state was not reached for Zn in the animals exposed to labelled soil (treatment A and B). Figure 3 depicts the development of ^{65}Zn concentration in one individual exposed to treatment B. For treatment C steady-state concentrations were reached within 6 to 8 days (Figure 4), and in treatment D within 12 to 14 days. The body residue of Zn taken up from soil in the isopods reached approximately 0.56 MBq kg^{-1} fresh weight in treatments A, B and D, while from a combination of soil and food (treatment C) the uptake reached a maximum of 40 MBq kg^{-1} fresh weight. Differences are mainly caused by the high label concentration in the food compared to the soil.

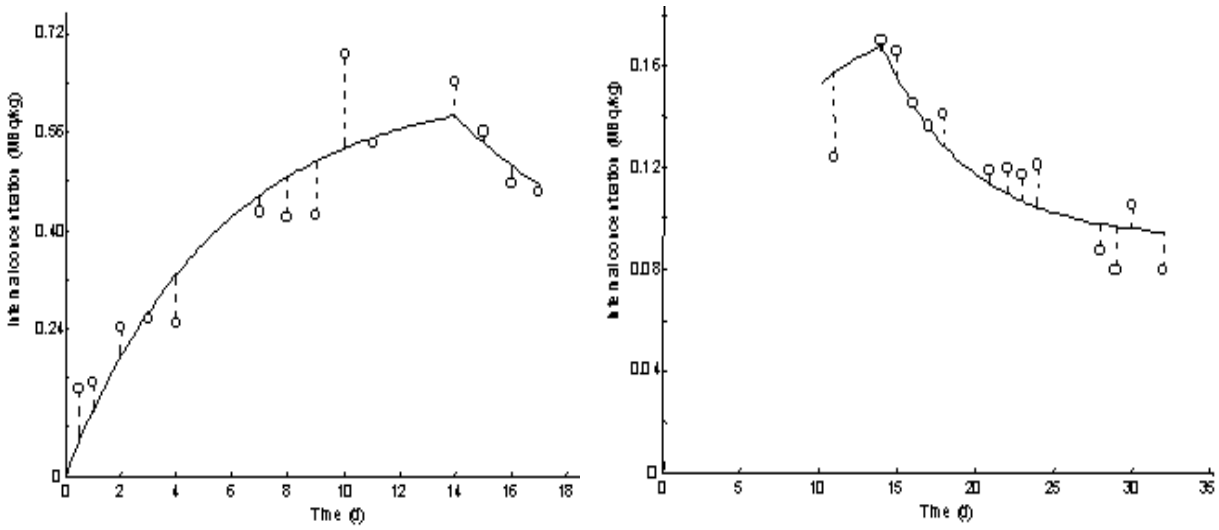

Figure 3: Accumulation of ^{65}Zn (in MBq kg^{-1} fresh weight) in *Porcellio scaber* exposed to ^{65}Zn labelled soil and unlabelled food (treatment B). Six individual animals were followed in time. As an example, one individual is depicted here during uptake and one during the elimination experiment.

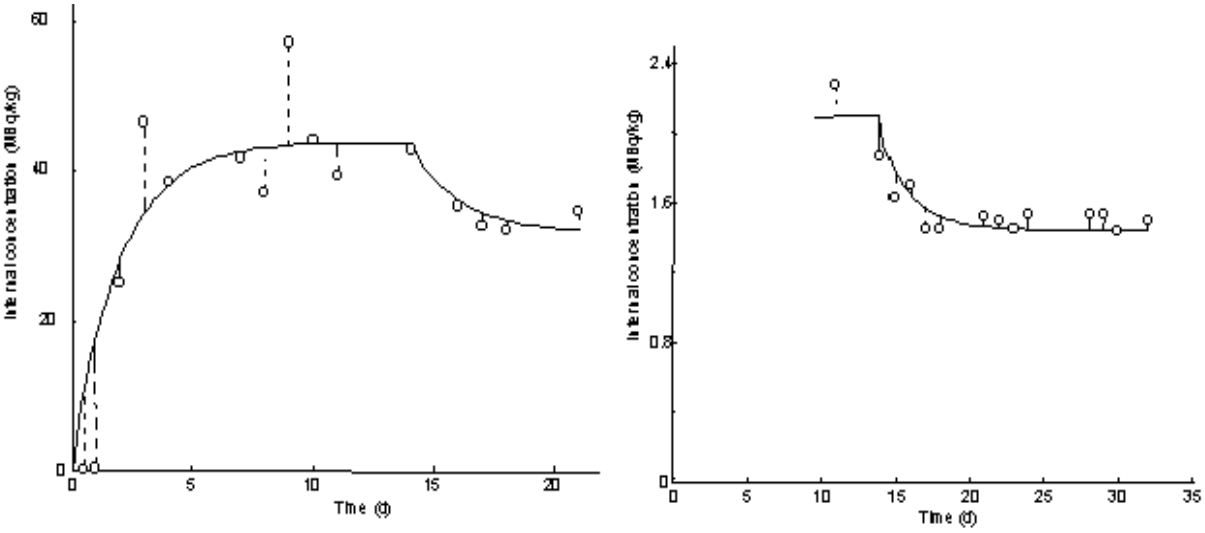

Figure 4: Accumulation of ^{65}Zn (in MBq kg^{-1} fresh weight) in *Porcellio scaber* exposed to ^{65}Zn labelled soil and ^{65}Zn labelled food (treatment C). Six individual animals were followed in time. As an example, one individual is depicted here during uptake and one during the elimination experiment.

After transfer to non-labelled soil and food, the ^{65}Zn concentration in the isopods decreased. Animals exposed only to ^{65}Zn labelled soil (treatment A and B) showed a relatively fast elimination (Figure 3). Animals exposed in treatment C showed a fast initial decline in internal levels ascribed to the refreshment of the gut content followed by elimination of ^{65}Zn during approximately three days. After four days, ^{65}Zn elimination stopped and concentration remained constant over time (Figure 4). This is explained by rather fast Zn turnover kinetics and by a relatively large storage of Zn in the inert fraction from which no elimination occurs. This is shown by the kinetic parameter values for Zn modelled from the accumulation patterns that are given in Table 4.

Table 4: Estimates of kinetic parameters for ^{65}Zn accumulation in six individual isopods, *Porcellio scaber*, exposed to the different treatments described in Tables 1. Uptake rate constants (k_1) from either food or soil were estimated for each individual separately, while the other parameters were assumed to hold for all animals. Uptake rate constants were derived from the uptake fluxes (a) by taking the external concentration into account. The k_1 ($g_{soil}\ g_{animal}^{-1}\ d^{-1}$) is the uptake rate constant either from soil only exposure ($k_{1'soil}$), from labelled soil and unlabelled food (k_{1soil}), or from unlabelled soil and labelled food (k_{1food}), k_2 (d^{-1}) is the elimination rate constant, Fi is the fraction stored in inert form and Cgut the content of the gut (MBq kg^{-1}). Minimum and maximum value are likelihood-based 95% confidence intervals.

	value	min-max		value	min-max
treatment A			treatment B		
k2	1.93	1.20 - 3.15	k2	0.182	0.10 - 0.31
Cgut	0.063	0.00 - 0.158	Cgut	0.00	0.00 - 0.029
Fi	0.09	0.06 - 0.18	Fi	0.55	0.41 - 0.67
k1' soil	0.398		k1 soil	0.048	
k1' soil	0.243		k1 soil	0.039	
k1' soil	0.252		k1 soil	0.058	
k1' soil	0.252		k1 soil	0.010	
k1' soil	0.175		k1 soil	0.016	
k1' soil	0.311		k1 soil	0.029	
treatment C			treatment D		
k2	0.507	0.35 - 0.78	k2	0.230	0.06 - 0.65
Cgut	0.117	0.00 - 0.396	Cgut	0.00	0.00 - 0.57
Fi	0.73	0.6 - 0.8	Fi	0.61	0.0 - 0.61
			k1 food	0.012	
			k1 food	0.009	

The selective feeding behaviour is reflected by the gut content dynamics. In treatment A, the gut was filled with soil, in treatment B the gut was filled with unlabelled food as shown by the concentration estimated to be zero. In treatment C, a significant amount of radioactivity came from the gut contents, which can predominantly be explained by labelled food and in combination with labelled soil. The gut content of animals exposed in treatment D, with unlabelled soil and labelled food, showed the best model fit when the gut content was zero, however, the confidence interval ranged to a maximum of 0.57 MBq kg^{-1}. These results are consistent and in agreement with the findings for Cd.

A remarkably high value for the elimination rate constant (k_2) was shown in treatment A, in which no food was added. For Zn, the k_2 was large in contrast to Cd, while k_2 for Cd in treatment A was lower than in the other treatments (see Table 3). This may be explained by the rather high uptake rate constant. In treatment A, uptake is only via the soil and Zn taken up is hardly stored in the inert fraction, as shown in Table 4. Therefore, it is conceivable that

the animal compensates for the high uptake from soil by fast elimination kinetics. The Zn elimination rate constants for the isopods exposed in the treatments B and C are different from each other (no overlap of confidence intervals). Zinc enters the animal's body in high amounts when exposed to labelled soil and labelled food, and this is compensated for by a higher elimination rate together with storage in the inert fraction. Fi values are 0.55 and 0.73 for treatment B and C respectively, but do no significantly differ from each other. The elimination rate constant (k_2) was not significantly different between animals exposed to labelled soil (treatment B) and labelled food (treatment D). Also the Zn retrieved from the inert fraction was similar in all treatments, except for treatment A.

Uptake rate constants (k_1) from either food or soil were derived from both uptake fluxes (a_{soil} and a_{food}) by taking the external concentration into account. For the animals exposed to treatment A, the mean uptake rate constant 0.272 ± 0.076 d^{-1} was approximately a factor 10 higher than for animals exposed to the treatments B and D. This efficient Zn uptake reflected in the $k_{1'soil}$ can be explained from the lack of food. Zn is a metabolically required element and uptake from soil gives a negligible amount of Zn stored in the body (Fi was low, see Table 4), therefore uptake efficiency should be high. The uptake rate constant (k_{1food}) derived for the animals exposed to treatment D was slightly lower compared to the uptake rate constant (k_{1soil}) for animals exposed to treatment B.

Non-radioactive Zn levels in sacrificed animals after 14 days exposure were 453 ± 156 mg kg^{-1} dry weight (n=4). The animals at the end of the experiments, after 32 days of exposure, contained; 365 ± 62.3 (n=3), 429 ± 85.3 (n=3), 422 ± 193 (n=3) and 336 ± 38.8 (n=2) mg Zn kg^{-1} dry weight, for treatment A, B, C, and D respectively. These levels are comparable to the concentration of 385 ± 92.5 µg Zn g^{-1} d.w. in the isopods before exposure.

5.4 Discussion

Food and soil taken up orally will enter via the oesophagus to the foregut where mixing with enzymes from the hepatopancreas takes place, from there the mixture passes to the hindgut. Digestion takes place in the anterior chamber of the hindgut and fluids are transferred via the typhlosole channels into the lumen of the hepatopancreas where nutrients are absorbed (Van Straalen and Donker 1994, Hames and Hopkin 1989). The digestive system of isopods makes it understandable that metals in food and soil (taken up orally) will mainly enter the hepatopancreas without even reaching other parts of the body. It is known that Cd in the hepatopancreas is mainly present in the S (small) cells, which consists of granules either containing sulphur or mainly composed of breakdown products. Zn in the hepatopancreas is mainly retrieved in the B (big) cells, which are rich in acid phosphatase, and in granules that are formed by deposition of calcium phosphate (Hopkin and Martin 1982, Hopkin et al. 1989). Once metals are incorporated in the sulphur-containing S-cells, they are excluded from circulation. Metals that enter the granules of the B-cells may possibly be excreted again, because these cells show daily cycles of apocrine excretion towards the lumen (Hames and Hopkin 1991a).

These metal-specific differences agree with the results of ^{109}Cd and ^{65}Zn kinetics of the isopods exposed to the different of treatments in our experiments. In general, Cd uptake flux

via both routes (soil and food) was rapid and the animal is transporting excess Cd as fast as possible towards storage (Fi) and excretion (see high k_2 value). Although Hames and Hopkin (1991b) did not measure elimination kinetics nor storage fractions, their results gave similar trends for *Porcellio scaber* exposed to ^{109}Cd labelled leaves. Zn taken up via the soil is not transported to the inert fraction, and as a consequence the inert fraction for Zn is relatively low compared to Cd and similar in all treatments. Zn can be rapidly excreted from the body.
This agrees with the internal transfer of Zn through the body, and the Zn translocation to the hepatopancreas described by Donker et al. (1996). Elimination to maintain internal Zn concentrations is also known from the literature. For instance, decapod crustaceans were shown to be able to regulate their internal Zn concentration via excretion (Rainbow 2002, Chan and Rainbow 1993, Rainbow and Kwan 1995). Donker et al. (1996) also found that regulation is controlled mainly by active excretion of Zn, with tolerant isopod populations having better-developed abilities for excretion than non-tolerant populations.

For both Cd and Zn, the elimination rate constant (k_2) was similar in all treatments except for the treatment without food (treatment A). This suggests that food passing the gut is stimulating metal elimination. Cd and Zn loosely-bound to the gut epithelium together with relatively free metals circulating through the body may be excreted via this route. Food offers an excess of sorption capacity by its high organic content, through which free or loosely bound metals can be captured and eliminated from the body.

The similarity between the k_{1soil} and k_{1food} for Cd is remarkable, since k_{1soil} includes Cd uptake via the gut route and via the pleopods. The similarity of the uptake rate constants (food and soil) suggests that the uptake route of metals via the pleopods is negligible compared to the oral uptake route and that the animal fills its gut according to its feeding preference. Furthermore, whatever is in the gut will enter the body with similar effectiveness. Focusing on the animal's specialized storage and elimination capacity, the uptake from soil and pore water resulted in slightly less storage in inert fractions (hepatopancreas), but this did not result in an increase of elimination capacity when comparing animals exposed to soil (treatment B) and to food (treatment D) (Tables 3 and 4). It might be that metals from the food move directly via the digestive tract into the hepatopancreas while metals taken up from the soil remain longer in the tract, to increase absorption possibilities of required elements, before they are translocated to the storage compartment.

Semi-mechanistical modelling
The metal uptake kinetics of isopods exposed to either soil or food were modelled by applying a one-compartment model (equation 1). For the elimination kinetics, a one-compartment model was used in which an inert fraction was incorporated (equation 2). We assume that this fraction exchanges with the rest of the body during accumulation, but becomes inert only during elimination. The use of a two-compartment model, in which Fi is not a fraction but a separate compartment (as an infinite sink) does not agree with the data, as such a model would not be able to produce steady-state body residues. Rather, this model

would predict constantly increasing internal concentrations. Even though our model offered only a partial mechanistic explanation, it appeared to be capable of describing the development of a steady state under continued exposure, combined with an internal inert compartment. The actual mechanism is likely to be more complicated than our model, e.g. parameters are under physiological control and therefore cannot be considered constant.

Quantifying uptake from combined exposure via food and soil

The simplified semi-mechanistic model used in this study fits the data accurately, and we therefore applied the parameters derived for the kinetics under single exposure conditions (soil or food; treatment B or D) for predicting metal uptake by isopods exposed to a combination of soil and food (treatment C). Calculating uptake fluxes into animals from combined exposure, using uptake rate constants derived in single exposure treatments, is allowed when the decrease of ^{109}Cd and ^{65}Zn activities due to radioactive decay is negligible and the isotopes act as stable metals. Measurements over time of non-radioactive and radioactive metal levels showed that this was the case in our experiment (data not shown, but for concentrations see Table 2). The mean uptake rate constants derived from the experimental treatments B and D were used to obtain the relative contribution of each individual uptake route in treatment C according to equation 3. Cd uptake flux was estimated as: $a_{soil} + a_{food} = 0.038 \times 12.6 + 0.014 \times 9.04 = 0.605$ mg kg^{-1}d^{-1}. The internal steady-state concentration, which is the ratio of this uptake flux and the elimination rate constant (0.258 d^{-1}; Table 3) equals 2.35 µg g^{-1}. Added to the initial Cd concentration of 5.05 ± 0.82 µg Cd/g d.w. (n=4), this gives 7.40 mg kg^{-1}, which is in agreement with the non-radioactive steady-state concentrations of 8.24 ± 1.7 µg Cd g^{-1} d.w. (n=4) measured after 14 days of exposure, just before the isopods were transferred to the elimination soil. From these results it can be concluded that soil and food uptake can be taken as additive and that total Cd uptake can be predicted accurately from the uptake and elimination rate constants obtained from single exposure routes. The elimination rate is independent of the uptake route and influenced by the Cd fraction stored inert, which does depend on the uptake route.

The same exercise was applied to compare calculated and measured Zn concentrations in the organisms exposed to food and soil. Initial non-radioactive Zn concentration in the isopods was 385 ± 92.5 µg Zn g^{-1} d.w. (n= 4). Uptake flux of Zn in isopods exposed to treatment C can be written as: $a_{soil} + a_{food} = 0.033$ (d^{-1}) × 2223 (mg kg^{-1}) + 0.011 (d^{-1}) × 606 (mg kg^{-1}) = 80.0 mg kg^{-1}d^{-1}. Together with the elimination rate constant (0.507 d^{-1}; Table 4) this equals 157.8 µg g^{-1}. Added to the initial Zn concentration, a steady-state concentration of 544 µg Zn g^{-1} d.w. is reached, which is comparable to the steady-state concentration of 453 ± 156 µg Zn g^{-1} d.w. (n=4) measured after 14 days of exposure.

5.5 Conclusion

Metal accumulation kinetics for the isopod *Porcellio scaber* exposed to either soil or food or their combination are the net result of uptake rate constants (k_1), elimination rate constant (k_2) and an inert fraction (Fi). For both Zn and Cd, the uptake kinetics expressed as fluxes derived from the single exposure experiments (either to food or to soil), worked in an additive way to

the metal accumulation in the isopod. The uptake rate constants for Cd upon soil and food exposure were of the same order of magnitude. Zn uptake rate constants from food were slightly lower than from soil. The Cd and Zn elimination ability differs with the uptake route due to the way the metal traffics through the isopod's body and is subsequently captured. This storage ability in inert fractions has consequences for the steady-state level that is reached. However, assuming equilibrium of the concentrations in soil and food, and considering that $k1_{soil}$ and $k1_{food}$ are similar and additive (see our results), it may be concluded that the relative importance of the uptake routes depends on the partitioning of metals between soil and food.

Acknowledgement

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Chapter 6

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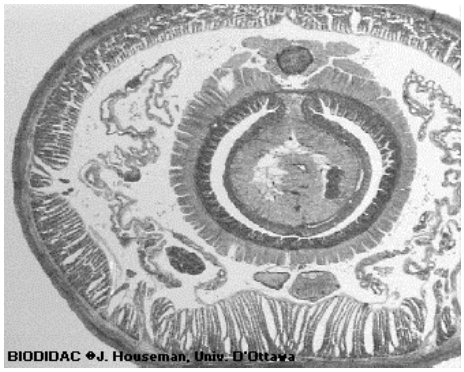

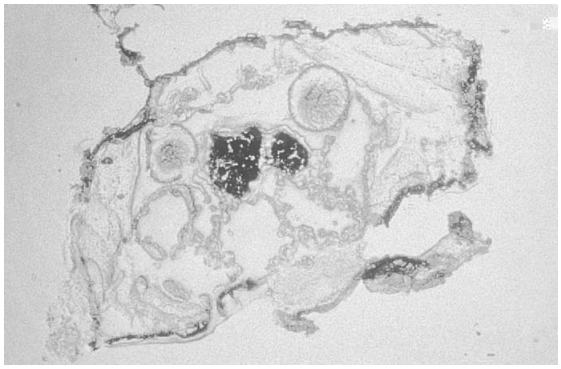

Chapter 6
Surface adsorption of metals onto the earthworm *Lumbricus rubellus* and the isopod *Porcellio scaber* is negligible compared to absorption in the body

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Abstract

In terrestrial organisms, bioaccumulation is usually based on a summation of the amount of metal adsorbed to the body wall and absorbed into the body. The relative proportions of metal adsorption and absorption are usually not quantified. In this study, the distinction between adsorbed and absorbed metals was investigated in two different terrestrial species exposed to metals for two weeks. The earthworm *Lumbricus rubellus* was chosen as representative for organisms mainly taking up metals via the dermal route, and the isopod *Porcellio scaber* as an organism taking up metals mainly via the alimentary tract. Cross-sections of whole animals were made using a cryostat and accumulated metals were localized by means of a phosphor screen (autoradiography). Radiolabels were used to determine the distribution of metals over the different organs and to distinguish between adsorption and absorption. Cd in the earthworm was mainly found in tissues of the chloragogenous region, whereas Zn was also found in various other organs and in the connective tissue. In the isopod, both Cd and Zn were mainly located in the hepatopancreas. Adsorbed amounts of Cd and Zn were negligible compared to internalized Cd and Zn concentrations for both organisms. Consequently, when focusing on effects of metal uptake for the organism itself, there is no need to correct for adsorption. This suggests that adsorption to the epidermis is not a rate limiting step in metal uptake by soil invertebrates.

6.1 Introduction

In conventional bioassays that determine metal accumulation, organisms are exposed to a contaminant in soil or water for a certain time-period. Collection of the animals from the experiment is usually followed by washing in water and blotting dry on filter paper. In the case of soil organisms, a short incubation period on filter paper and food abstinence is often included, to depurate the gut content. Subsequently, the organisms are sacrificed by freezing and analyzed for body metal concentrations. In practice this means that the sum of externally adsorbed and internally absorbed metals is considered (Langston and Spence 1995), and it remains unclear whether this is justified.

In mechanistically based uptake experiments, the uptake rate is often described in more detailed steps, based on a widely accepted concept of metal uptake, the Free Ion Activity Model (FIAM) (Campbell 1995). Distinction is made between diffusion of metals from the bulk solution to the biological surface, and membrane passage. In water-rich environments, the first step is generally believed to be rapid (Garnham et al. 1992). The actual uptake of metals into the organism, the absorption, is kinetically slow. Metal uptake is controlled by the net electronegative charge of the membrane that is governed by polar pores (Foulkes et al. 1993). However, not all accumulated metal burden is necessarily absorbed into the living cells and tissues in the body. A proportion of the metals may bind to extracellular structures composed of collagen, chitin, other fibrous materials, mucus etc. Adsorption of metals onto the cuticle and mucus is therefore defined as an extracellular process.

Metal adsorption rates to surface structures have been quantified in several studies. Surface metal adsorption onto organisms is studied by washing the outside of the biotic epidermis with a chelating agent, thereby removing metals from the outside but not affecting internalized metals. A one-minute EDTA wash (0.02 and 0.2 M solution) performed on the bacterium *Rhodospirillum rubrum* exposed to soluble Cd for 13.5 minutes, showed a large extractable (adsorbed) Cd fraction compared to a small non-EDTA-extractable internalized Cd fraction (Smiejan et al. 2003). From Zn accumulated in the fresh water oligochaete *Tubifex tubifex* 90% could be washed off with EDTA-solution (Fleming and Richards 1981). For Pb, adsorption on the algae *Chlorella vulgaris* was washed off using a one-minute 0.01 M EDTA-solution, and a constant amount of adsorbed Pb was found already after 10 minutes of exposure, while internalized Pb showed a slow linear increase (Slaveykova and Wilkinson 2002). This indicates that adsorption is time dependent and, in the long run, becomes less important compared to absorption. This statement can be made for all uphill transport mechanisms of ions in cells, regardless of the species (Wolterbeek and Verburg, 2002).

For small aquatic organisms, metal uptake is relatively fast. For terrestrial invertebrates it is hypothesized that uptake takes more time, compared to small aquatic species, due to smaller contact area (with water) for aquatic species. Additionally, the surface/volume ratio in microinvertebrates, usually used in aquatic studies, is smaller in the macroinvertebrates that are used in terrestrial studies, resulting in faster uptake rates. For the terrestrial earthworm *Eisenia fetida*, both absorption and adsorption were quantified using an IDA-chelator: 80% was attributed to adsorption. Fleming and Richards (1982) concluded that the external mucus layer adsorbs significant quantities of Pb and Fe and serves as a barrier to the rapid entry of

these metal ions. Compared to aquatic organisms, the mucus layer on the epidermis of terrestrial organisms usually is thick to protect the animal from damage, and might be rate limiting in metal uptake kinetics (McLaughlin 2001). However, the conclusion of Fleming and Richards (1982) needs some caution, since a short exposure time (one hour) was applied and extremely high metal concentrations were used.

Obviously, to quantify the relevance of adsorption compared to absorption, exposure time should be much longer than the time required to saturate the external barrier with metals. When using longer uptake periods, adsorption may already be at steady state, while absorption is still continuing and progressively increasing in relative importance. In conclusion, there is a need to distinguish adsorption and absorption over soil organisms in a realistic exposure period.

In this study, two-week exposure experiments were conducted with two different terrestrial invertebrates using radiolabelled metals. In soil ecosystems, two groups of invertebrates, namely soft-bodied and hard-bodied organisms, are distinguished (Vijver et al. 2003a). The first group of species consists of organisms with a highly permeable cuticle; the earthworm *Lumbricus rubellus* belongs to this group. Accumulation in earthworms can best be described based on soluble metal pools in the soil (Peijnenburg et al. 1999, Saxe et al. 2002, Vijver et al. 2003b). The second class involves species having a less permeable cuticle, to which the isopod *Porcellio scaber* belongs. The isopod cuticle consists of chitin hardened by calcium salts (Ruppert and Barnes 1994). Accumulation in hard-bodied organisms can best be described on the basis of total metal pools in soil (Vijver et al. 2003a), due to the major importance of oral ingestion of soil constituents.

Both test species were exposed for 14 days in ^{109}Cd labelled or ^{65}Zn labelled soil or fed with ^{109}Cd labelled or ^{65}Zn labelled food. Whole body autoradiography was applied to cross-sections, aimed to qualify adsorption and absorption of Cd and Zn. This technique allows for metal localization in the organism's body at the tissue and organ-level. It was hypothesized that organisms predominantly exposed by food have less adsorption to the cuticle and epidermis than organisms exposed via the dermal route.

6.2 Material and methods

Earthworms and isopods were collected from a non-polluted clay-rich grassland soil and a non-polluted sandy garden soil, respectively and kept in soil with high clay content, under conditioned laboratory conditions at 15°C with constant light. Isopods were given shelter by placing some stone fragments on the soil surface. A two-week experiment was carried out, in which three test organisms were exposed via soil or food. A two-week exposure period was shown to give a significant metal accumulation in both test species (Vijver et al. 2005, Vijver et al. submitted). Moreover, a two-week exposure period is sufficient to cover initial uptake and to reach a more or less constant ratio between adsorption and absorption. Adult earthworms of an average (± st dev, n=3) fresh weight of 342.5 ± 111.3 mg and adult isopods of a average (± st dev, n=3) fresh weight of 47.5 ± 14.6 mg were used for the experiment.

Soil and food were single labelled with ^{109}Cd or ^{65}Zn at loads given in Table 1, by adding a stock-solution with chloride salts as counter ion. Radioisotopes were supplied as $^{109}CdCl_2$

(Amersham Biosciences, Buckinghamshire, UK) and $^{65}ZnCl_2$ solution (Perkin Elmer, Boston, USA). The soil was collected from the floodplain Ruitersplaat in the Biesbosch, The Netherlands, having 17-19% organic matter, 22-28% clay, and a pH (0.01 M $CaCl_2$) of 7.5-7.9. The soil was kept at a moisture content of 80% w/w (= 72% of maximum Water Holding Capacity). Food was supplied *ad libitum* to the organisms and consisted of soaked poplar leaves *Populus x canadensis*.

Table 1: The actual radioactivity counts ± standard deviation (expressed in Becquerel gamma radiation) of ^{109}Cd or ^{65}Zn in the labelled soil (n=6) and food (n=3) used in the earthworm and isopod exposure experiments.

	Soil (Bq/gram)	Food (Bq/gram)
^{109}Cd	9,263 ± 170	602,251± 36,008
^{65}Zn	1,757 ± 42	164,504± 11,700

^{109}Cd and ^{65}Zn loads in soil, food and organisms were determined using a Wallac gamma counter (Model 1480 3, EG&G company, Finland).

Cross-sections of earthworms and isopods

From each exposure treatment, one earthworm and one isopod were sacrificed without gut depuration using liquid nitrogen. Approximately 600 cross-sections with a thickness of 14 µm over a selection of the organism' body were made using a MICROM cryostat (type Cryo-Star HM 560 M, Germany) at a temperature of –15°C. The sections were air-dried and directly used for autoradiography analyses. Sections were made in different areas of the body in order to visualize sites at which ingestion, digestion and secretion activities and resorption of essential nutrients and non-essential contaminants take place. The earthworm *L. rubellus* was cut at four regions: in 1) the oesophagus near segment 10; 2) the anterior intestine between segments 20 and 25; 3) the mid intestine between segments 25 and 44; 4) the posterior end of the hindgut, behind segment 45. For the isopod *P. scaber* three regions were defined: 1) the foregut; 2) the midgut; 3) the hindgut or so-called papillate region.

Autoradiography and light microscopy

^{109}Cd and ^{65}Zn accumulated by the exposed organisms emit β and γ rays, which may cause ionizations in a photo-stimulable phosphor layer (PSP). Focusing on γ rays, the ionization process causes electron/hole pairs in the PSP crystal. The stored energy (in the form of trapped electrons) forms a latent image, imprinted on the exposed PSP layer. Stimulation of the traps and release of stored electrons was performed by local energy deposition using a highly focused laser light source. In the used BaFBr:Eu phosphor screen, released electrons result in the emission of a 3 eV photon which can be read by a micro-channel plate detector (Packard Cyclone, USA) and appear as black and grayish spots. To image the cross-sections for morphology a light microscope at a magnification 7 * 2.5 (Zeiss, West Germany) was used. The two micrographs of a cross-section were then overlain, to allow assignment of metal depositions to tissues.

6.3 Results

Exposed organisms appeared healthy and the individuals showing the highest accumulation using whole body measurements (Wallace gamma counter), were sacrificed for cross sectioning. Control organisms did not give signals above detection limit, and therefore no autoradiography images were made. As use of radioactive label only aimed at metal localization, no quantification of the label was made.

After exposure in soil, both the earthworm and the isopod showed sufficient accumulation of Cd to make detection possible. Uptake of Zn from soil by both organisms could not be detected. This can be explained by the low soil loads (Table 1). In addition, localization of Zn was aggravated by the detection efficiency on the phosphor plate, which is higher for β-emission than for γ-emission. After calibration the low gamma of ^{109}Cd appeared more detectable than the hard beta and high gamma of ^{65}Zn.

For earthworms, uptake via the food was low for Cd. For isopods feeding on ^{109}Cd-labelled food, accumulation was sufficient for accurate detection. Uptake of Zn via the food was high for isopods, and lower but still sufficient for detection in earthworms.

Metal distribution in earthworms

Autoradiography results of the earthworm exposed to ^{109}Cd-labelled soil, or fed with ^{65}Zn-labelled food are summarized below (Figures 1 – 3). Note that no results on earthworms exposed to ^{109}Cd labelled food and ^{65}Zn labelled soil could be obtained.

1) Oesophagus

Cd was located in the ingested particles and scattered in the calciferous glands and vesicles, some parts of the coelomic fluid, connective tissue and the longitudinal muscle (results not shown). The micrograph for Zn was not clearly developed, and no results can be described.

2) Anterior intestine

Cd was mainly located in the gut, which is considered as external Cd. Internal Cd was found in the chloragogenous tissue (Figure 1). Cd was not found in the epidermis and in very low amounts in the body wall musculature. The micrograph for Zn was not clearly developed and no results can be described.

3) Mid intestine

No Cd adsorption to the epidermis was found. Internalized Cd could be located in the intestinal epithelium, typhlosole, and in chloragogenous tissue (Figure 2). Some Cd spots could be detected over the cross-section and near the longitudinal muscles. Zn was found evenly distributed and was present in various tissues, such as the longitudinal muscle tissue, peritoneum and in the coelomic fluid. Zn was also detected in the gut, gut epithelium, and chloragogenous tissue. Zn adsorption on the epidermis was not detected (results not shown).

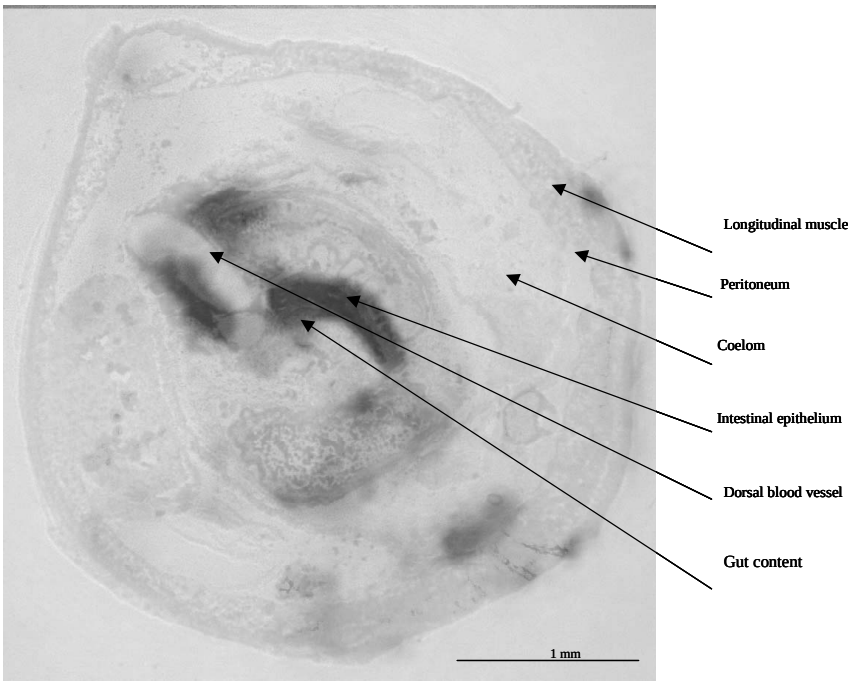

Figure 1: Cd in the anterior intestine of the earthworm *Lumbricus rubellus* exposed to ^{109}Cd-labelled soil. The dark shadings overlain on the photographs originate from the radiolabels that were detected.

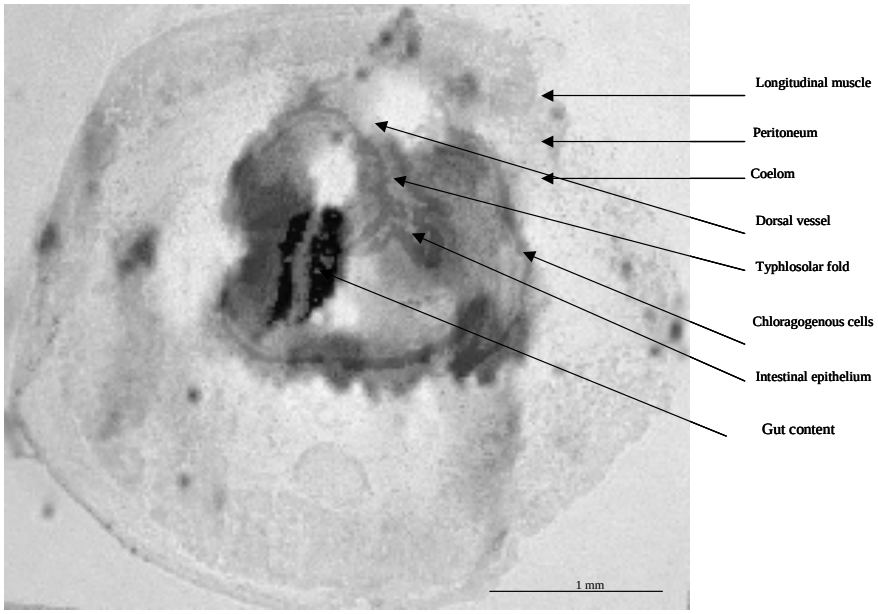

Figure 2: Cd in the mid intestine of the earthworm *Lumbricus rubellus* exposed to ^{109}Cd-labelled soil. The dark shadings overlain on the photographs originate from the radiolabels that were detected.

4) Posterior intestine

Large amounts of external Cd were located in the lumen of the intestine. No epidermal adsorption of Cd nor internalized Cd was found (results not shown). Zn was detected in the gut content, and not adsorbed on the epithelial wall of the intestine. Internalized Zn was found in the longitudinal muscle tissue, the peritoneum, the coelomocytes, the gut epithelium, and chloragogenous tissue (Figure 3).

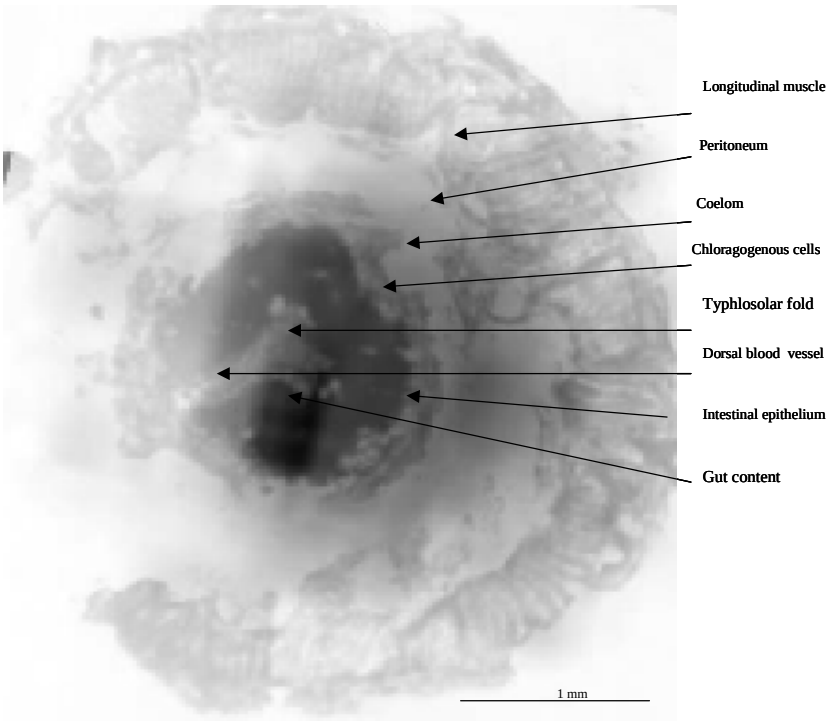

Figure 3: Zn in the posterior intestine of the earthworm *Lumbricus rubellus* fed with ^{65}Zn-labelled food. The dark shadings overlain on the photographs originate from the radiolabels that were detected.

Metal distribution in isopods

Autoradiography results of the isopod exposed to ^{109}Cd-labelled soil, exposed to ^{65}Zn-labelled soil, or fed with ^{65}Zn-labelled food are summarized below (Figures 4 –6). Note that no results on isopods exposed to ^{109}Cd-labelled food could be obtained.

1) Foregut

All Cd was concentrated at the periphery of the gut and in tissue adjacent to the gut (results not shown). Zn was found in the gut. Internalized Zn was located in tissue around the gut, and immediately below the gut, maybe at the place where the typhlosole channels start to develop (Figure 4).

2) Hindgut, anterior region

The gut content contained Cd. At the external surface a high Cd load was found, which was probably contamination when sampling from the soil. Internalized Cd was located in hepatopancreas and gut epithelium (results not shown). In an isopod exposed to Cd-labelled food, Cd was located in three out of the four hepatopancreas tubules, in the gut epithelium and externally in the gut (see Figure 5). Zn was found in the gut and in three out of the four tubules of the hepatopancreas. In tissue around the hepatopancreas, a little Zn could be detected (results not shown).

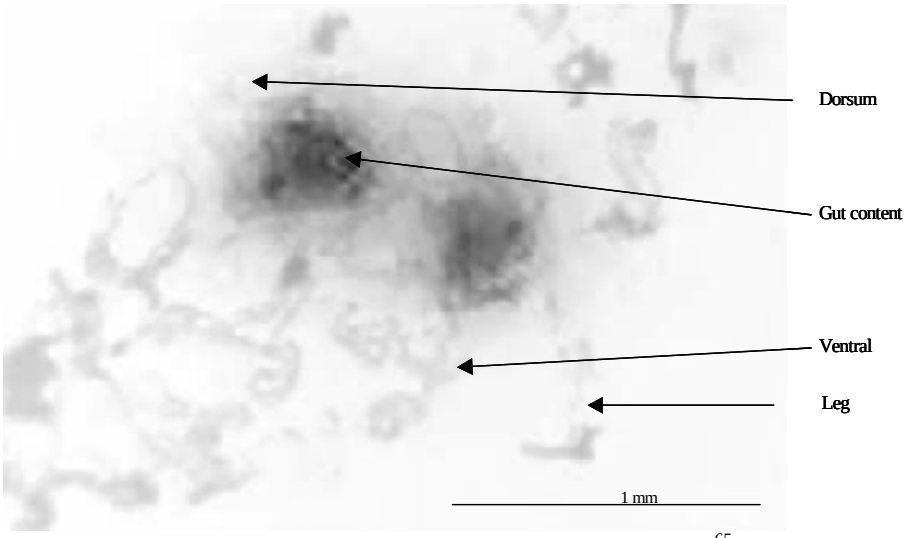

Figure 4: Zn in the foregut of the isopod *Porcellio scaber* fed with ^{65}Zn-labelled food. The dark shadings overlain on the photographs originate from the radiolabels that were detected.

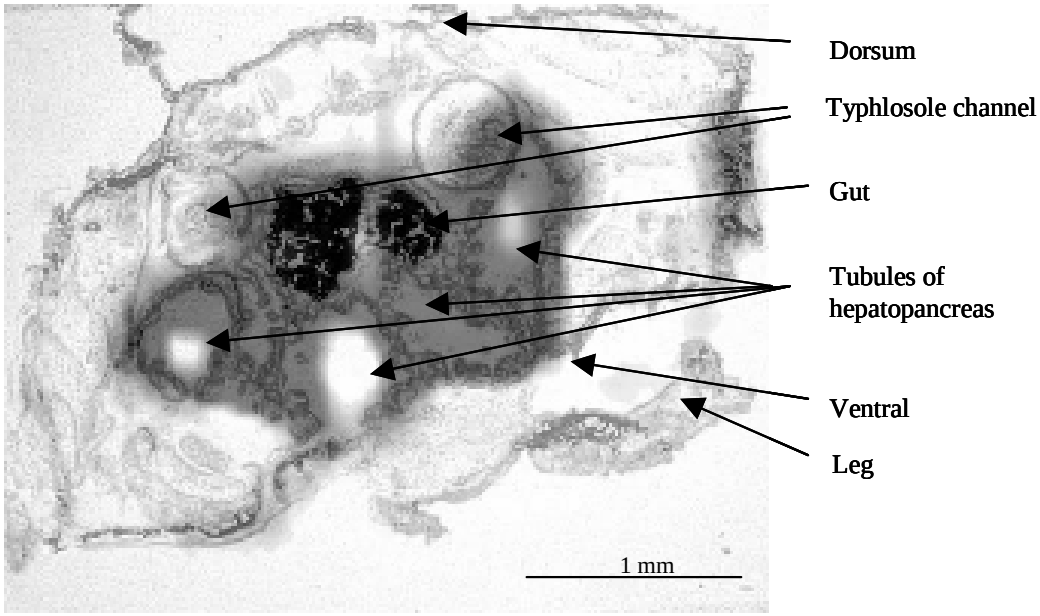

Figure 5: Cd in the anterior region of the isopod *Porcellio scaber* fed with ^{109}Cd-labelled food. The dark shadings overlain on the photographs originate from the radiolabels that were detected.

3) Hindgut, papillate region

Cd was found in the periphery of the gut and in tissue adjacent to the gut (Figure 6).

Zn levels in this part of the body did not exceed the detection limit, therefore results could not be described.

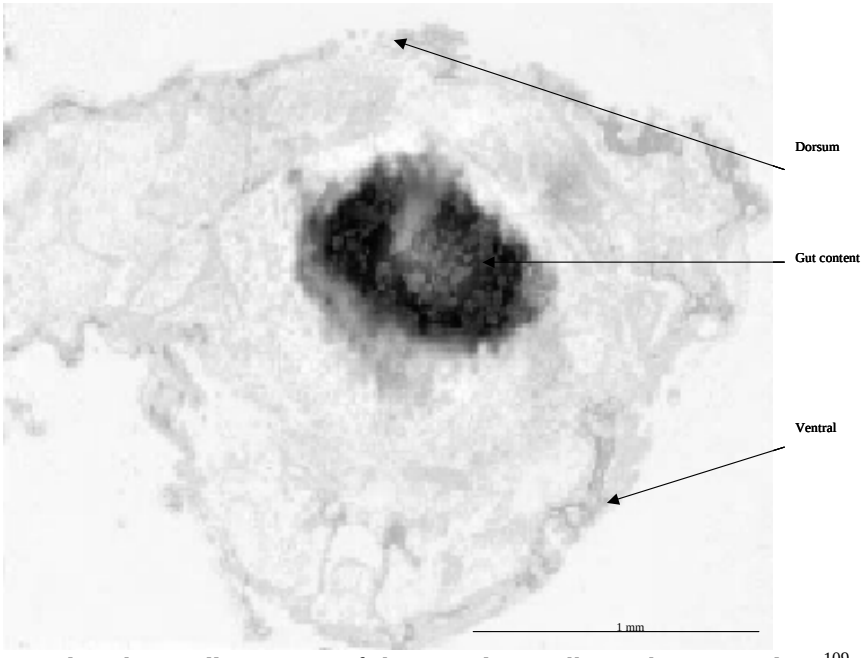

Figure 6: Cd in the papillate region of the isopod *Porcellio scaber* exposed to ^{109}Cd-labelled soil. The dark shadings overlain on the photographs originate from the radiolabels that were detected.

6.4 Discussion

Metal distribution in earthworms

Autoradiography photographs showed no adsorption of ^{109}Cd or ^{65}Zn on the cuticle or the epidermis of the earthworm exposed to either food or soil. This is in agreement with the findings using a chelator method to quantify adsorbed metals on the epidermis of the earthworm *Lumbricus rubellus* after 14 days exposure to a metal-rich floodplain soil (unpublished results P. van Vliet and M.G. Vijver). It was concluded that after washing with various concentrations of EDTA (ranging from 2.5 mM - 0.02 M) the internalized metal concentrations did not differ from those in organisms not washed. Metal levels in the EDTA solution were below the detection limit. From using either the EDTA wash or the autoradiography method, it may therefore be concluded that metal adsorption to the earthworm epidermis is negligible.

Ireland and Richards (1981) reported that 68% of the Cd in *Lumbricus rubellus* was associated with the surface mucus, but this amount could, however, not be removed by washing. This Cd localization in the epidermis is in disagreement with findings for the earthworm *Eisenia fetida,* for which relatively low concentrations were found in the body wall compared to other body tissues (Prinsloo et al. 1999). The Cd spots near the longitudinal muscles (Figure 3) might be identified as nephridia, which are known to accumulate Cd (Prinsloo et al. 1999). Like our results, most authors report metal localization in the chloragogenous tissue (Morgan and Morgan 1990), which is unique to oligochaetes and has a function in metal storage and trafficking (Stürzenbaum et al. 2001). This tissue is packed with intracellular granules containing high amounts of sulphur, phosphorus and calcium. Elevated metal levels can reduce the volume of granules and concomitantly increase the volume of debris granules (Morgan et al. 2002).

Zn was distributed over various tissues, including muscles and coelomocytes. This can be attributed to its essential role in membranes, enzymes and proteins that are main constituents of muscles, which explains its wide distribution over the body. Zn was also found in the granules of the chloragogenous tissue. It is known that granules not only act as storage for all metals, when essential metals are involved they also function as buffer for the coelomic fluid and blood (Stürzenbaum 1997).

Metal distribution in isopods

No metals were adsorbed to the cement-like exoskeleton of the isopod exposed via soil or via food. This can be explained from the suspected low binding affinity of metals to the exoskeleton. The Cd distribution throughout the body was the same for isopods exposed via food and via soil. In isopods, the hepatopancreas appears to be the most important storage organ for metals (Hopkin and Martin 1982, Donker et al. 1990, Witzel 1998). Like the chloragogenous tissue in earthworms, the hepatopancreas in isopods is packed with different types of granules. In these granules, metals are precipitated in different chemical forms (Hopkin et al. 1989). It is obvious that organs with digestive, storage or excretory functions are the prime sites for localization of granules (Brown 1982, Köhler 2002).

Zn was mainly located in tubules of the hepatopancreas, and not visible all over the cross-section. This can be explained by the anatomy of the isopod, because organs like the typhlosole and hepatopancreas are directly associated with the gut. The respiratory system and the reproduction organs are situated at the ventral side of the skeleton, near the hindgut. Metals in these organs may be transported through the haemolymph.

6.5 Conclusion

We hypothesized that animals that take up metals via the dermal route (e.g. earthworms) show relatively more metal adsorption onto the epidermal membrane than organisms predominantly exposed via the oral route (e.g. isopods). However, for both the earthworm *Lumbricus rubellus* and the isopod *Porcellio scaber*, the results showed that Cd and Zn adsorption to the surface was negligible. This suggests that it is not likely that adsorption to the body wall, and subsequent passage through the membrane, is the rate-limiting step for metal uptake in these terrestrial invertebrates over a longer period of time. The preliminary results elicit many questions on metal adsorption being important for metal uptake kinetics, which only can be answered by more detailed scientific research.

Acknowledgement

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Chapter 7

Internal metal sequestration and its ecotoxicological relevance – a review

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Paracelsus

Chapter 7
Internal metal sequestration and its ecotoxicological relevance – a review

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Abstract

Organisms are able to control metal concentrations in certain tissues of their body to minimize damage of reactive forms of essential and nonessential metals and to control selective utilization of essential metals. These physiological aspects of organisms are not accounted for when assessing the risk of metals in the environment. The Critical Body Residue (CBR) approach relates toxicity to bioaccumulation and biomagnification, and might at first sight provide a more accurate estimation of effects than the external concentration. When expressing CBRs on total internal concentrations, the capacity of organisms to sequester metals in forms that are not biologically reactive is neglected. The predictability of toxic effects will increase when knowledge on metal compartmentalization within the organisms' body is taken into account. Insight in metal compartmentalization sheds light on the different accumulation strategies organisms can follow upon metal exposure. Using a fractionation procedure to isolate metal-rich granules and tissue fragments from intracellular and cytosolic fractions, the internal compartmentalization of metals can be approximated. In this review, current knowledge regarding metal compartmentalization in organisms is summarized, and metal fractions are identified that are indicators of toxicity. Guidance is provided on future improvement of models, such as the Biotic Ligand Model (BLM), for risk assessment of metal stress to biota.

7.1 Introduction

A general principle in pharmacology is that the concentration of a chemical at the receptor determines its effect. In ecotoxicology, this principle is translated into the critical body residue (CBR) concept, and is used in the Biotic Ligand Model (BLM) and in bioaccumulation monitoring for risk assessment. In the CBR concept, the total body concentration is assumed to be proportional to the concentration at the target or receptor. The CBR is defined as the threshold concentration of a substance in an organism that marks the transition between no effect and adverse effect. The CBR approach integrates internal transport and metabolism processes and toxicity at specific sites of toxic action (McCarty and Mackay 1993, Hickie et al. 1995). Tedious assessment of chemical availability as modified by environmental characteristics is thus overcome. CBRs have been successfully developed for a wide range of nonpolar narcotic organic chemicals. The concentrations for acute mortality are relatively constant (2-8 mmol/kg wet weight) between groups of chemicals with similar mode of action and between different organisms (McCarty and Mackay 1993, McCarty et al. 1992). Also for organic compounds having a specific mode of action, for mixtures of chemicals having similar modes of action, and for other (sublethal) endpoints, such as reproduction effects, CBRs have been determined (McCarty and Mackay 1993, Fitzgerald et al. 1997, Parkerton and Konkel 2000, Fay et al. 2000).

Comparison of body concentrations with CBRs may be an effective tool for site specific risk assessment of toxicants (Lanno et al. 1998, McCarty 1997, Van Wensem et al. 1994), but for metals this does not seem to work as accurate as with organic compounds (Van Straalen 1996). The problems can be found in the mechanisms underlying metal accumulation and toxicity that are more complicated than for organic compounds and are species-specific (McCarty and Mackay 1993). This was e.g. demonstrated by Crommentuijn et al (1994), who found rather large differences in lethal body concentrations for Cd in different soil invertebrate species.

In case of metals, toxicity cannot succesfully be predicted from total external dissolved concentrations. The free ion concentration is shown to correlate best to toxicity (Campbell 1995), but competition with other cations, such as Ca, Mg and protons, also affects uptake and toxicity (Osté et al. 2001, Plette et al. 1999, Van Gestel 1997). The latter aspect, together with the pharmacological principle mentioned above, formed the basis for the recently developed BLM. The BLM assumes that the effect is proportional to the concentration of metal bound to the target site, and that this target site (biotic ligand) is in direct contact with the external (aquatic) environment (Paquin et al. 2002, Di Toro et al. 2001). The BLM uses chemical speciation modelling and seems well capable of describing acute toxicity of metals for fish, when the gill is the target site. Although recently also some BLMs have been developed for other aquatic organisms, such as crustaceans and algae (De Schamphelaere and Janssen 2002, Heijerick et al. 2002), it remains unclear whether the assumption that the target is in direct contact with the external environment is always valid. Recent studies (e.g. Niyogi and Wood 2003) demonstrate that chronic exposure may modify the gill-metal binding characteristics. It therefore remains unclear whether gill-metal binding constants derived in

BLMs for acute toxicity may also be applicable for predicting effects of long-term metal exposure.
The problems with using the CBR approach for metals, and related problems mentioned to its use in the BLM and bioaccumulation monitoring, demonstrate that a better understanding of the internal comparmentation of metals in organisms and its consequences for toxicity is required. It is apparent that there is a need for partitioning the total metal body burden, because similar to the external concentration, only a portion of total body burden is biologically available for interaction with sites of toxic action. The major reason for studying sub-cellular metal partitioning is to better understand mechanisms of accumulation and toxicity. This review therefore aims at:

- providing an overview of mechanisms used by organisms to compartmentalize metals;
- identifying the ecotoxicologically relevant metal fraction in organisms;
- identifying a pragmatic and relatively simple method to be used in practice to determine ecotoxicologically relevant metal fractions inside organisms.

We hypothesize that differences between species in critical body concentrations of metals reflect different internal compartmentalization strategies, and that the fraction causing toxic effects may be similar for all species. Similarities in metal metabolism by aquatic and terrestrial invertebrates and fish facilitate the comparison of metal compartmentalization and no distinction is made in this review between these organisms. From this point of view, internal fractionation resembles external metal speciation and aims at determining the internal bioavailable concentration.

7.2 Metal accumulation strategies

A diversity of specific metal accumulation strategies is known. The physico-chemical properties of metals and the physiology of the organism both influence metal uptake, distribution, tissue accumulation, and excretion (Depledge and Rainbow 1990). A general physiological pattern of metal trafficking through the organism's body is depicted in Figure 1. An internal metal pool required for normal metabolism can be distinguished from a metal pool above the metabolic requirements and which is stored in various forms (Rainbow 2002). The capacity of the metabolically reactive pool has an upper limit, and toxicity is related to (an overflow of) this pool rather than to the total internal metal burden.

Rainbow (2002) distinguished different metal accumulation strategies for essential and nonessential metals. Essential metals may be subject to regulation either by limiting metal uptake at the level of the total body concentration, or by involving organism-specific accumulation strategies with active excretion from the metal excess pool and/or storage in an inert form and/or excretion of stored (detoxified) metal. For nonessential metals, excretion from the metal excess pool and internal storage without elimination are the major strategies, and body concentrations generally increase with increasing external concentrations. Although this illustrates the diversity of metal accumulation strategies at the level of organs and tissues in an organism, tissue and organ-specific metal accumulation are ultimately determined by cellular mechanisms.

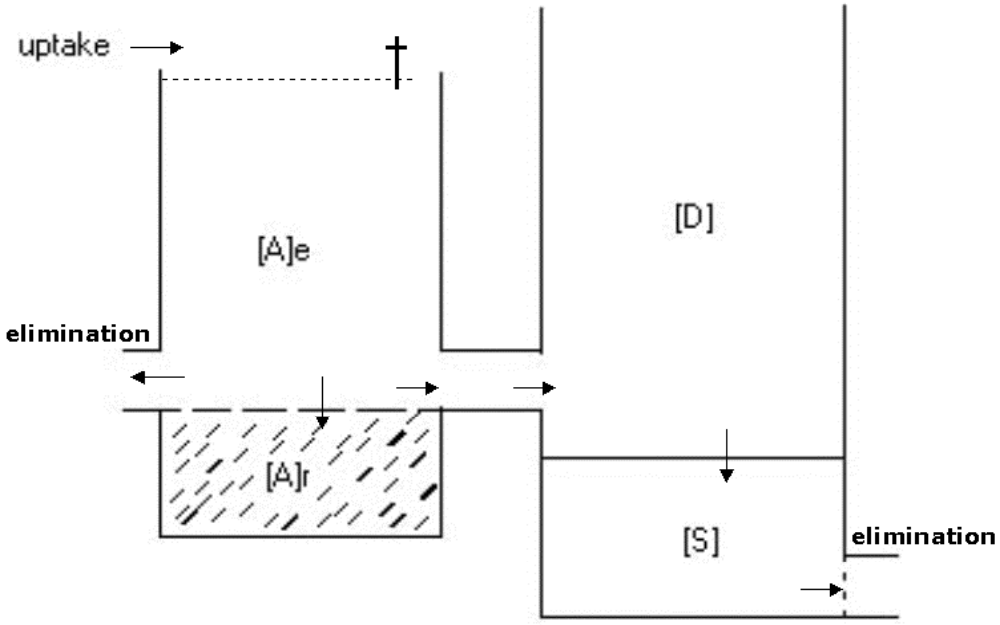

Figure 1: Generalized scheme showing the various compartments in which metals may be present and accumulate inside an animal (modified from 22). [A]r is metabolically metal pool, for nonessential metals this pool is negligible; [A]e is excess pool above metabolic requirements, causing toxicity and eventually mortality when elimination or detoxification fluxes are slower than uptake rate; [S] is storage; [D] is detoxification.

At the cellular level, biotic systems have evolved control mechanisms to minimize accumulation of reactive metal species and to facilitate optimal utilization of essential metals. This is achieved by the various chemical forms in which metals can be present in an organism, including:

- free ionic form or complexed ion species (e.g. $CdCl_2$, $CdCl^+$, $CdCl_3^-$);
- bound in the active centre of functional proteins (e.g. hemoglobin, hemocyanine) and low molecular weight peptides (e.g. zinc finger proteins);
- bound in the active centre of enzymes (e.g. cytochromes, carbonic anhydrase, superoxide dismutase);
- bound to low molecular weight organic acids (e.g. citrate);
- bound to metallothionein, to transport proteins (e.g. ferritin), or other sequestration proteins;
- bound in vesicles of the lysosomal system, as intracellular granules;
- precipitated in extracellular granules, mineral deposits, residual bodies and exoskeletons;
- bound to cellular constituents potentially causing dysfunction (enzymes, ion channels, DNA).

Metal ions in excess of metabolic requirements and storage capacity are potentially toxic and must be removed from the vicinity of important biological molecules. Therefore, the capacity of internal sequestration has a huge impact on the sensitivity of an organism to metals. Two major types of cellular sequestration, known to occur after increased exposure to metals, can affect their toxicokinetic availability to organisms (Cherian and Nordberg 1983). The first one

involves the formation of distinct inclusion bodies, the second one the binding of metals to heat-stable proteins. Hopkin et al. (1989) demonstrated metal accumulation in inclusion bodies in isopods using X-ray microanalysis. Different granule types were distinguished: Type A - amorphous deposits of calcium phosphates, accumulating e.g. Zn; Type B - granules originating from the lysosomal system and containing mainly acid phosphatase, accumulating e.g. Cd, Cu, Hg and Ag; Type C – excess iron stored in granules as haemosiderin (Hopkin 1989). Mineral granules or concretions have been identified in intestinal cells of many invertebrates, including Collembola (Humbert 1977, Van Straalen et al. 1987) and Crustacea (Brown 1982). Calcium/phosphate or sulfur-rich organelles, functioning as waste nodules, were found in Oligochaeta (Andersen and Laursen 1982). In earthworms, the phosphate-rich structures, named chloragocytes, are metal-sequestrating organelles that respond to elevated metal exposure by increasing the volume of the debris vesicles and thereby decreasing the chloragosome volume (Morgan et al. 2002). In this way, efficient plasticity in metal storage capacity can be obtained.

The second mechanism of cellular sequestration preventing metal toxicity is a cytoplasmic process involving a specific metal-binding protein, metallothionein. Metallothioneins (MT) are low molecular weight, cysteine-rich, metal-binding proteins that occur throughout the animal kingdom, as well as in plants, eukaryotic microorganisms and some prokaryotes (Nordberg and Kojima 1979). The induction of MT by a variety of metal ions, including Cd, Cu, Hg, Co, and Zn, has led to their frequent use as a biomarker for the presence of elevated levels of metals in the environment. Many types of stimuli can induce MT production, not only including essential and nonessential metals, but also hormone-like compounds, and environmental stress factors (Korsloot et al. 2004). This wide range of inducibility, which is generally believed to indicate that MT plays a pivotal role in the regulation of many processes, compromises the use of MT as a specific response to metals. Most studies in which MT induction by metals took place were carried out at relatively high metal concentrations in laboratory settings. It is unclear to which extent this phenomenon is likely to occur in nature. Vallee (1979) summarized the evidence for the involvement of MT in limiting cell injury. Basically, the availability of toxic metals is reduced by binding to MT. Verbost et al. (1989) concluded that cells with a limited capacity to synthesize MT and a low cellular turnover rate are expected to develop severe disturbances in their Ca homeostasis when exposed to Cd^{2+}. The MT is located mainly in the cytosol, but can also be found in the nucleus in amounts dependent upon the tissue metal concentration (Panemangalore et al. 1983). Affinity for MT is metal-dependent, and correlated with the distribution of metal binding sites on the MT as well as the stoichiometry of the different types of MT. These differences in binding strength are relevant for the involvement of MT in metal-metal interactions. For essential metals, MT may be important in controlling metabolically available concentrations by binding the metals in a non-toxic unavailable form until they are required for various metabolic processes.

7.3 Compartmentalization of metals in biota

Sub-cellular metal partitioning is the basis of internal metal sequestration over different organs and tissues. The compartmentalization or sequestration of metals by invertebrates is

dependent upon many factors, such as metal-type, organism life history and metal pre-exposure. The way organisms deal with metals differs and possibilities are outlined:

Metal-dependency

The metabolic pathways and internal compartmentalization of metal ions with closely related chemistry - such as Cd and Zn - remain distinct, even when they are elevated within the same environment (Morris et al. 1999). Differences in compartmentalization of different metals can be ascribed to their specific ionic radius and electronegativity. Metals like Cd, Cu and Pb mainly have affinity to nitrogen or sulphur-containing groups. Other metals, like Ca, Al and Be, are more effective at binding with oxygen-containing groups, such as carboxylic acids and alcohols. The third group of metals, including Ni and Zn, has no binding preference and will form ligands with many functional groups; these are the so-called borderline metals (Nieboer and Richardson 1990). These chemical properties have effects on the compartmentalization of metals.

In the midgut gland of snails (*Helix pomatia*) from unpolluted sites, Zn was evenly distributed between soluble and particulate fractions (centrifugation at 27,000 g), while Cu, Cd, and Pb were distributed in a ratio of approximately 3:1. After feeding the snails Zn-spiked lettuce, Zn concentrations remained constant in the soluble fraction, whereas levels in the particulate fraction increased. Cd concentrations in both fractions increased, with a larger increase in the soluble fraction. Cu concentrations remained evenly distributed between soluble and particulate fractions, likely due to the role of Cu in the respiratory pigment, hemocyanin. Pb was observed primarily in the particulate fraction (Dallinger and Wieser 1984). Fractionation of whole earthworms (*Lumbricus terrestris, Allolobophora chlorotica, Dendrobaena pygmea*) resulted in a similar distribution of metals. Cd was preferentially retrieved in soluble fractions obtained after centrifugation at 100,000 g, Cu prevailed in the pellet fraction and Zn was evenly distributed over both fractions. For Cd and Zn, this distribution was not affected by exposure of the earthworms to 100 mg/kg Cd spiked in soil, while Cu was more evenly distributed over both fractions in exposed earthworms (Rouabah and Descamps 2001).

Pre-exposure or tolerance-induction

External concentration and duration of exposure had significant effects on the distribution of Cd in the deposit-feeding worm *Limnodrilus hoffmeisteri* (Wallace and Lopez 1996). The amounts of Cd bound in the soluble fraction, obtained after centrifugation at 10,000 g, increased with time and exposure concentration, whereas Cd concentrations in cellular debris [in more recent studies referred to as Metal Rich Granules, MRG] remained at a relatively constant level. In a later study, Wallace et al. (1998) found that *L. hoffmeisteri*, collected from a severely Cd-polluted site, produced metallothionein-like proteins (MT) as well as MRG for Cd storage and detoxification after exposure. Worms collected from an adjacent unpolluted site only produced MT after Cd exposure. The fraction sensitive to metal exposure, i.e., organelles and heat-stable proteins, was considered a labile pool that influenced the recovery from adverse exposures. Release of Cd and Zn from MRG was found to be negligible

(Wallace et al. 2003). These results suggest that MRG plays a role in tolerance to long-term exposure while MT mainly act to protect against short-term Cd exposure.

Stürzenbaum et al. (2001) characterized two isoforms of metallothionein, wMT-1 and wMT-2, in *Lumbricus rubellus*. The isoform wMT-1 was suggested to have a dominant role in metal regulation. Worm MT-2 probably is the primary detoxifying ligand and its concentration in the earthworms was related to Cd exposure in a dose- and time-dependent manner. Worm MT-2 is located in vesicles of the chloragocytes, and most likely transports Cd towards the MRG. Waste nodules, in which metals were detoxified and stored, could be identified in the posterior portion of earthworms using atomic absorption and X-ray fluorescence spectrometry (Andersen and Laursen 1982). Metals are strongly bound in these granules and release only occurs with death of the earthworm and subsequent lysis of cells. This supports the finding of Wallace et al. (1998) that MRG play a role in metal detoxification.

Honeycutt et al. (1995) examined Cd distribution in earthworms after exposure on filter paper or in Cd-spiked artificial soil. Upon dissection, Cd was mainly found within the intestinal tract of the earthworm. The soluble fraction obtained after centrifugation at 100,000 g contained the highest amount of Cd (40- 63% of the total Cd). Some 30% was recovered from the pellet obtained after 10,000 g centrifugation, while the pellet fraction obtained after centrifugation at 100,000 g (microsomes) contained approx. 10%. Both in soil and on filter paper, the soluble fraction increased with time while the pellet fraction decreased with time. This suggests translocation of Cd through the cell membrane into the cytosol, where metallothionein has the ability to detoxify the incoming Cd by binding.

Using centrifugation at 10,000 g, Conder et al. (2002) also defined two fractions of internal Cd compartmentalization in earthworms exposed to a Cd concentration as high as 14.0 mmol/kg (approx. 1575 mg/kg) in artificial soil (see Figure 2). Here the supernatant fraction of the earthworm digests increased linearly throughout the 14 d exposure period (from approximately 0 to 3.59 mmol/kg), whereas the pellet, containing the MRG and tissue fraction, reached an average steady-state concentration of 1.2 mmol/kg. Apparently, upon short-term exposure, the MRG has a limited capacity to storing incoming metals.

In springtails, granular structures found in the gut epithelium have a strong affinity for metals and are proposed to play a role in metal regulation and excretion. Storage of Cd and Pb in the gut epithelium and excretion during moulting have been reported (Van Straalen et al. 1987). Between moults, approximately 90% of the total Cd burden in the species *Orchesella cincta* is present in the gut epithelium, from which about 50% is excreted by intestinal exfoliation during moult (Posthuma et al. 1992). Metal excretion in metal-tolerant springtails is more effective than in non-tolerant organisms. Hensbergen et al. (1999) identified and characterized MT in the springtail that was only induced by Cd exposure and not by Zn exposure (Sterenborg et al. 2003). The metabolic pathway in the springtail is thought to involve transfer of MT-bound metal to the vesicles of the lysosomal system. When new epithelial cells are formed, unbound Cd is resorbed which again induces MT, or MTs are resorbed from the old gut epithelium while Cd is fixed in granules. Subsequently, the old gut is excreted as a gut pellet (Sterenborg 2003). This demonstrates that MRG not only plays a role in Cd sequestation, but may also contribute to excretion.

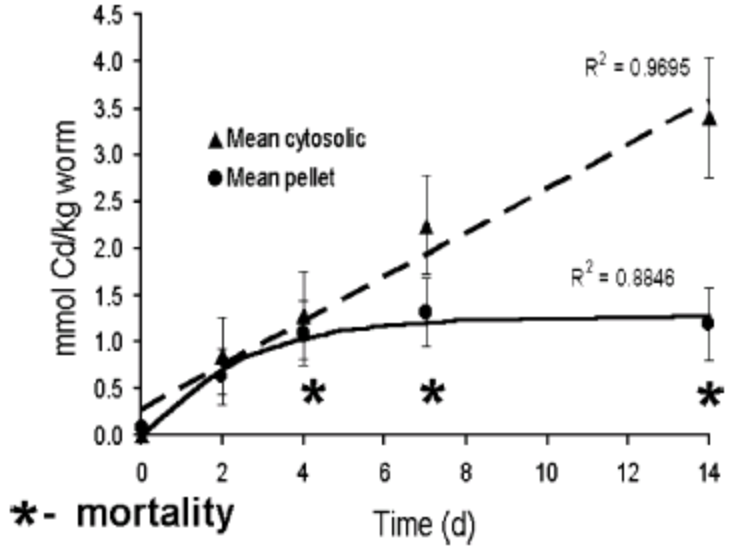

Figure 2: In earthworms exposed to 1575 mg Cd/kg soil, the whole body concentration is separated into a cytosolic fraction that increased in time and a pellet fraction that reached steady state (from Conder et al. 2002, with permission by Elsevier). As soon as the storage capacity was reached, the supernatant fraction became proportionally larger and toxicity was seen.

For isopods, production of metal-binding proteins alone could not explain the differences in sensitivity between populations from unpolluted and polluted sites (Donker et al. 1996, Dallinger and Prosi 1988), but as far as we know no MT has been identified in isopods. Isopods efficiently store metals in the hepatopancreas, resulting in low metal concentrations in the body fluid (Donker et al. 1996). The storage capacity in the hepatopancreas of isopods contains high-density granules to which metals are bound (Clifford and Witkus 1971). When metal storage capacity of these granule-containing hepatopancreas cells is exceeded or when storage cannot keep up with uptake rate, isopods will suffer metal toxicity (Hopkin 1990) (see also Figure 1).

Species-dependency

Janssen et al. (1990) found rather large differences in cadmium concentrations in soil arthropods collected at the same site, and no relationship could be found with trophic position. To further investigate this phenomenon, Janssen et al. (1999) determined accumulation and elimination kinetics in four different arthropod species. Large differences were found, which could mainly be attributed to differences in accumulation strategies. To predict the toxicological consequences of this finding, Crommentuijn et al. (1994) determined time-related cadmium toxicity in six soil arthropod species in relation to accumulation strategy. For all animals LC50, expressed as the Cd concentration in the diet causing 50% mortality, decreased with prolonged exposure time. For animals having the possibility to excrete cadmium, such as the Collembola *Orchesella cincta* and *Tomocerus minor* and the oribatid mite *Platynothrus peltifer*, LC50 reached a constant value after a certain period of time, suggesting an equilibrium between uptake and elimination rates. LC50 kept on decreasing with increasing exposure time for animals not capable of eliminating cadmium, such as the isopods *Oniscus asellus* and *Porcellio scaber* and the millipede *Cylindroiulus brittanicus*. In

these cases, the concept of external LC50 breaks down, because a steady state is never reached, however the internal LC50 (LBC) still exists and can be estimated. Crommentuijn et al. (1994) found that although taxonomically-related species showed comparable Cd accumulation patterns, lethal body concentrations ranged between 37 and 4580 µg/g dry body weight. This shows that CBRs are species-specific, even for related species. Hopkin (1990) and Hames and Hopkin (1991) found substantial differences in metal accumulation between the isopod species *Porcellio scaber* and *Oniscus asellus*. Where *Oniscus asellus* was able to eliminate Zn, *Porcellio scaber* was not. This difference may be related to the differences in the relative abundance of B (big) and S (small) cells of the hepatopancreas. B cells appeared to be subjected to a daily cycling ejecting their content in the lumen of the hepatopancreas, whereas S cells remained more constant in number and shape. This suggests that B cells play a role in metal excretion and S cells in metal storage (Hames and Hopkin 1991).
In the fish *Perca flavescens* exposed to metals along a gradient of metal contamination due to smelter operations in Quebec, Cd and Cu accumulated predominantly in the heat-stable-protein fraction, whereas in bivalves, *Pyganodon grandis*, collected from the same area, metals accumulated primarily in the granules fraction (Giguère et al. 2003). Differences in metal accumulation patterns in two clam species, having different feeding habits, could especially be explained from the metal concentration in the cytosolic fraction (Wallace et al. 2003). The MT fraction played a major role in the metal excretion ability of the facultative deposit-feeding clam, which resulted in 22 times more Cd and 2 times more Zn accumulation in the filter-feeding clam exposed to the same medium. Rouabah and Descamps (2001) found that species-dependency of Cd uptake in different earthworm species can mainly be ascribed to the soluble fraction obtained after centrifugation at 100,000 g. *Lumbricus terrestris* reached the highest steady state levels based on total body burden, followed by *Allolobophora chlorotica* and *Dendrobaena pygmea*, but in all three species the soluble fraction contained most of the accumulated Cd. This shows that related species have similar metal compartmentalization, but the absolute amount of metal accumulated differs between species.

Relevance to trophic transfer in invertebrate food chains
The various internal metal fractions all have their own binding capacity for metals, which has implications for food-chain transfer to higher trophic levels. A study on the relationship between subcellular Cd distribution in an oligochaete and its trophic transfer to a predatory shrimp showed that only metal present in the soluble fraction (organelles and protein fraction obtained after centrifugation at 100,000 g) of prey is available for the predator. Factors influencing the subcellular distribution in the prey will directly alter trophic transfer to predators. In a recent paper, Wallace et al. (2003) showed that differences in subcellular distribution of Cd between resistant and nonresistant worms directly affected Cd availability for the predatory shrimp. When offered resistant worms, shrimp absorbed roughly 4 times less Cd than fed nonresistant worms (Wallace et al. 1998). Similar conclusions were found in a study using bivalves as prey, where the metal partitioning to organelles, denaturated proteins and metallothioneins (MT) comprise a subcellular compartment that is “trophically available” to predators (Wallace et al. 2003).

In studies examining the consumption of metal-contaminated isopods by spiders Dysdera crocata, Hopkin et al. (1989) also found that not all metal fractions are equally available to higher trophic levels. Metals bound in granules of the hepatopancreas of woodlice were not absorbed by the predatory spider, but metals bound to ferritin (Type C granules) were released and became available for uptake.
From the above, it may be concluded that metals in MT and tissue fractions were available for trophic transfer, while metals in MRG fractions were only partially available.

Link to uptake routes
Metal compartmentalization in organisms may depend on the primary route of uptake, through the diet or across the epidermal surface, because uptake routes influence not only the total uptake but also the sequestration of metals. In the lobster *Nephrops norvegicus*, Cd and Hg taken up via food were mainly accumulated in the hepatopancreas, and mainly in the gills and hepatopancreas after uptake from seawater (Canli and Furness 1995). Rainbow trout (*Oncorhynchus mykiss*) accumulated more Cd upon dietary exposure than after water-only-exposure. In water exposed fish, Cd mainly accumulated in the carcass, while in food-exposed fish most Cd was found in the intestinal tissue (Szebedinszky et al. 2001). Similar results were observed by Miller et al (1993) in trout exposed to differing levels of dietary and waterborne Cu. Not only did the route of uptake influence subsequent tissue Cu distribution among organs, but pre-exposure to waterborne Cu also induced a much greater tolerance to waterborne Cu in acute toxicity tests than dietary Cu pre-exposure, suggesting a physiological partitioning of Cu as well.
In isopods, Zn absorption involves two routes. First, Zn taken up from the food is transported to the hepatopancreas, directly from the gut fluid or via the typhlosole channels (Hames and Hopkin 1991). Second, Zn absorbed via other routes diffuses into the haemolymph, is partly excreted via the kidneys and partly accumulated in the hepatopancreas (Donker et al. 1996). Cd uptake and internal distribution did not differ in *Chironomus staegeri* larvae whether exposure was short-term (5 days) in water only or long-term (140 days) in water and sediment. In both cases, most Cd was stored in the midgut (Craig et al. 1998), probably also in granules.
Redistribution of metals to other tissues after the initial absorption phase has been reported to take place (Wright 1980, Marigomez and Ireland 1989, Vesk and Byrne 1999). Any fraction causing toxicity may only be present transiently. After chronic exposure, internal distribution will reach equilibrium and become independent of the exposure route. Conversely, the route of exposure cannot be identified based solely on the internal compartmentalization of metals in the organism's body.
As mentioned above, many organisms possess MT, which act as first scavenger of incoming metals. In some organisms, this MT is directly stored or excreted, while in other organisms, such as crustaceans, it is further processed and the metal is stored in granules, most likely Type B granules. The induction of MT is a rapid process, typically occuring at a time scale of several hours, whereas the encapsulation of metals in granules takes longer. Time-dependency

therefore is a factor that should not be neglected when studying internal distribution of metals in organisms.
It can be hypothesized that independent of uptake routes, dermal and oral, the size of the sub-cellular metal fraction that causes actual toxicity may be the same. The distribution over the fractions can differ with the uptake route, resulting in differential toxicity from the two routes of uptake.

7.4 Methods to analyze internal compartmentalization

From the above, it may be hypothesized that not the total body concentration but rather a fraction of it, is responsible for toxic effects in terrestrial and aquatic invertebrates. In order to apply the CBR approach for metals, it is necessary to establish a dose-response relationship between metal levels in an internal metal compartment and whole organisms responses. To this end, a procedure for the isolation of the various metal fractions is needed. It should be realized that fractions relevant to toxicity may differ from those relevant to metal accumulation. Here, some pragmatic approaches described in the literature will be evaluated. The pragmatic tools used originate from molecular biology and have in this way been validated, however, the operationally-defined fractions have to our knowledge not been examined microscopically to confirm their composition.
Honeycutt et al. (1995) determined Cd concentrations in different earthworm tissues and in different internal fractions. Coelomic fluid was collected from the whole organism by exposing the earthworms to ethanol. Thereafter the animal was fixed and the intestinal tract was dissected from the body wall. At the subcellular level, earthworms were homogenized in KCl followed by centrifugation at 10,000 g and 100,000 g, separating cytosolic fractions from microsomal fractions. Rouabah and Descamps (2001) used similar techniques, and so did Conder et al. (2002), Giguère et al. (2003) and Wallace and co-workers (1996, 1998, 2003). The differences can mainly be found in the number of centrifugation steps. The latter two researchers used the most elaborated protocol for the fractionation of organisms, an example is shown in Figure 3. Wallace & Lopez (1996) homogenized biotic samples followed by several centrifugion and/or digestion steps in which hydrolysis and protein separation were used. This fractionation method separates metal-rich granules (MRG) and tissue fragments from intracellular (nuclear, mitochondrial, and microsomal) and cytosolic fractions (i.e., metallothioneins (MT) and heat sensitive proteins).

The conceptual idea behind this fractionation protocol is based on the principles of metal speciation in soil or water, although obviously other types of binding occur in the biota as compared to the speciation reactions in the external environment. The underlying idea is that metals entering the body in a reactive form, are first captured by reversible (labile) binding to proteins and other ligands, followed by localization to targets that have a stronger affinity for metals. Metal exchange probably is slow in MRG and more rapid with MT and other proteins.

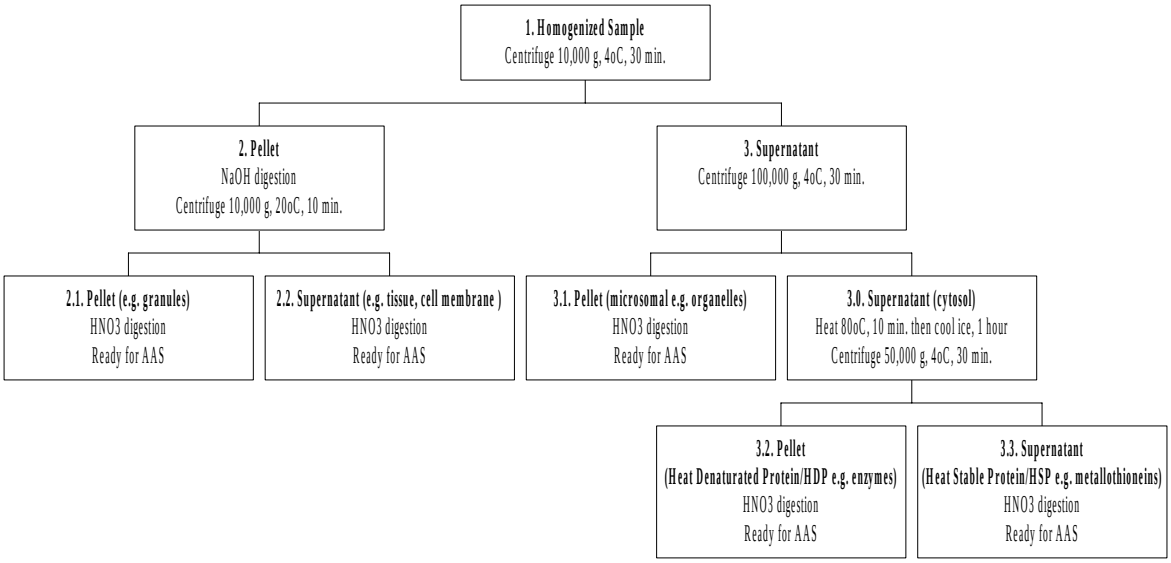

Figure 3: Protocol for internal fractionation of the metal residues in organisms (modified from Wallace and Lopez 1996). The fractionation separates the total body burden into five fractions of which only 2.2, a part of 3.1 and 3.2 are considered to be potentially toxic.

In general this idea is valid, although when testing metal compartmentalization in fish along a gradient (from low contamination to high contamination) it seemed that the distinction is not that sharp (Giguère et al. in prep). Fish collected from the low contaminated site of the gradient showed nonessential metals in the metal-sensitive fractions. This seems contrary to the hypothesis that nonessential metals are effectively detoxified in fish but above a certain threshold metal concentration these metals will "spill over" into potential metal-sensitive fractions causing adverse effects.

Fractionation methods mainly consist of different centrifugation steps (Figure 3), all reflecting the metal-binding preferences by different ligands. However, not all fractions can be linked directly to a certain organelle function. Most difficult to interpret is the microsomal/lysosomal fraction (pellet fraction obtained after centrifugation at 100,000g). On the one hand, if the lysosomal portion contains the metal, then it would be indicative of metal storage for eventual elimination. On the other hand, when metals are found in the microsomal fraction, it can be indicative of toxicity, since microsomes consist of smooth endoplasmatic reticulum, and contain many membrane-bound enzymes, responsible for e.g. protein synthesis and transport (Giguère et al. in prep). It should be realized that this fractionation protocol aims at a pragmatic separation of fractions. But it does not explain the biochemical processes underlying the metal accumulation strategies shown in Figure 1.

7.5 Synthesis

Just because a metal is available for uptake by an organism, it does not mean that it will be harmful. Metals can accumulate in organisms to high levels yet be isolated metabolically and not be toxicologically bioavailable. The biological significance of accumulated metal concentrations will be dependent on the way organisms cope with metal exposure. Figure 4 depictures the internal metal fractions that can be identified in organisms.

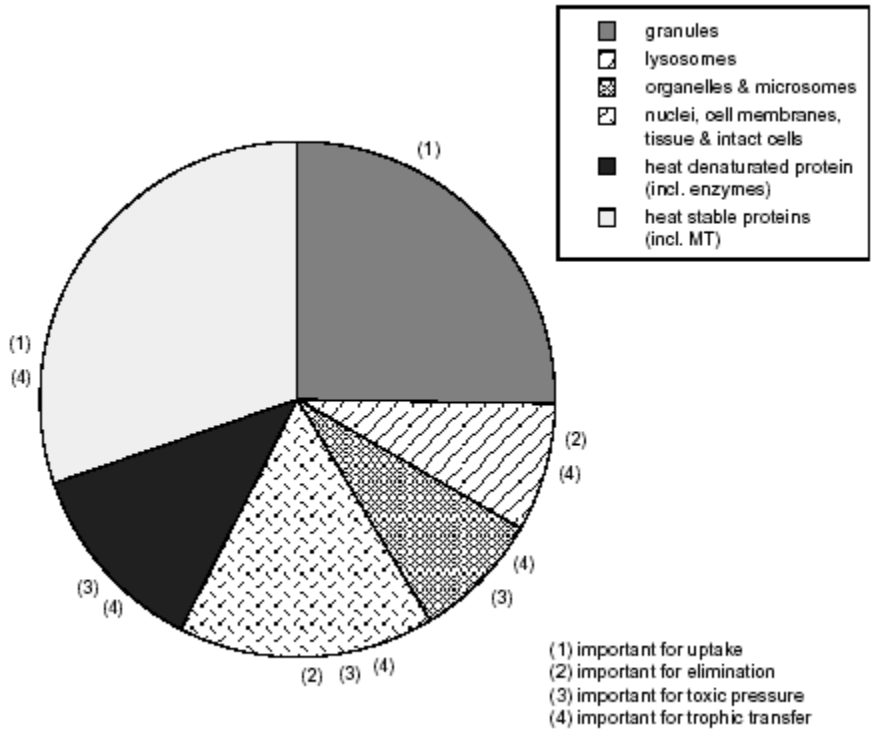

Figure 4: General hypothesis concerning the importance of internal metal fractions in organisms for the description of accumulation patterns, toxicity and trophic transfer. The size of fractions displayed corresponds to the distribution of Cd commonly found in organisms.

Understanding metal compartmentalization may explain accumulation and loss patterns in organisms. Metals retrieved from the excess pool (Figure 1) may easily be eliminated from the body, resulting in a saturation-type uptake curve. Storage in an inert fraction, that cannot or only slowly be eliminated from the body, however, will result in linear uptake patterns.

Upon short-term exposure to metals, organisms will induce metal-binding proteins, such as MT. As long as the organism produces MT, a detoxification process is active. Upon chronic exposure, storage in granules (also termed MRG) will play an important role in protecting the animal against metal stress. Elimination from these granules proceeds only slowly if at all.

The amount of metal compartmentalized in cytosolic, denaturated protein and tissue fractions seems to be most indicative of toxic pressure (Figure 4). These fractions are supposed to be indicative of the excess metal fraction shown in Figure 1.

Not all metal fractions are likely to be trophically transferred along the food chain. Especially the fraction stored in granules is hypothesized not to be available for assimilation by a (invertebrate) predator (Figure 4).

Models used to predict ecological risks of metals to aquatic organisms, such as the BLM, are based upon metal speciation in solutions, and were successful in predicting the acute toxicity of metals to aquatic organisms, such as fish (Meyer et al. 1999). In fish, the gill is usually the site of toxic action in acute metal toxicity. BLM therefore assumes that metal concentrations bound to the gill are proportionally linked to toxicity. For organisms other than fish, the BLM concept is using the theoretical foundations and assumptions of the CBR approach (De Schamphelaere and Janssen 2002), which involves that total body residues directly determine

the effect. And the total body residue in organisms is assumed to be directly related to the amount of metal bound to the external body surface. At the moment, the predictability of the BLM for the tested organisms is adequate, however, the applicability is restricted to small organisms that quickly achieve an internal equilibrium for the metals due to a large ratio between body surface and content. In case of macrofauna, the relationship between toxicity and total body residue is, however, not straightforward due to the internal compartmentalization of metals.

Another assumption of the BLM is that toxicity is driven by exposure to dissolved metal alone, rather than combined effects from dissolved and dietary exposures. The latter may be more relevant for chronic effects and for terrestrial organisms. Two groups of organisms are involved: 1) animals with a moist epidermis, that are able to take up metals across the skin and from the diet, and 2) animals with a well-developed cuticle, where dietary (food and drinking water) uptake predominates.

Szebedinszky et al. (2001) showed affinity changes of Cd on the gill of rainbow trout after 30 days of exposure to food and water. The log $K_{Cd\ gill}$ for water exposure was 6.54 and for the food exposure 5.92, while control fish gave log $K_{Cd\ gill}$ of 7.05. Therefore the log K value of metals determined from acute exposures is not representative for chronic exposures. Additionally, it is questionable if the accumulated concentrations on the gill can be indicative for sublethal and chronic effects due to differences in importance of uptake routes.

Methods that explain the underlying mechanistic and empirical relationships for the internal distribution of metals in biota can reveal the importance of internal metal concentrations. When assessing effects, it is insufficient to consider whole body metal concentrations without knowledge of tissue concentrations within the organism. After uptake of the metal (transport across the plasma membrane), the “free” reactive metal will circulate through the body fluid. Transport proteins will bind the metals reversibly and transport them into different compartments, where they are detoxified or not. Finding an indicator fraction proportional to the occurrence of effects is deemed necessary for essential and nonessential metals. The fractionation procedure outlined in Figure 3 may be a first step towards a practical tool to identify a fraction that is suitable to explain most of the variability observed in metal accumulation and toxicity in organisms.

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Chapter 8

Bio-classification of metals derived from sub-cellular partitioning in earthworms

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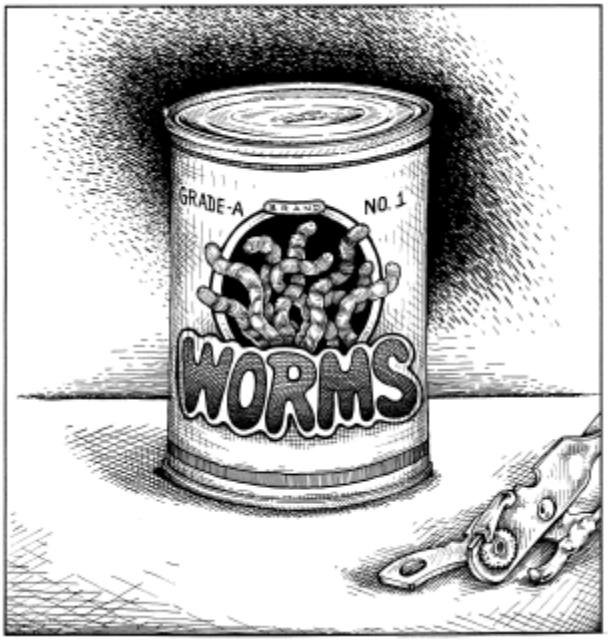
Submitted



Chapter 8
Bio-classification of metals derived from sub-cellular partitioning in earthworms

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Abstract

Metal ions in excess of metabolic requirements are potentially toxic and must be removed from the vicinity of important biological molecules to protect organisms from adverse effects. Therefore, metals are sequestrated in various forms, which may affect the accumulation pattern and the magnitude of steady-state levels reached. To investigate the sub-cellular fractions over which the metals Ca, Mg, Fe, Cu, Zn, Cd, Pb, Ni and As are distributed, earthworms *Aporrectodea caliginosa*, collected from the field, were analysed by isolating metal-rich granules (MRG) and tissue fragments from intracellular microsomal and cytosolic fractions (i.e., heat-stable proteins (HSP) and heat-denaturated proteins (HDP)). The fractions showed metal-specific binding capacity. Cd was mainly retrieved from the protein fractions. Cu was equally distributed over the protein fraction and the fraction consisting of tissue fragments, cell membranes and intact cells. Also Zn, Ca, Mg, and As were mainly found in the latter fraction. Pb, Fe and Ni were mainly isolated from the granular fraction. This bio-classification of metals might be useful in identifying the biological significance of steady-state levels reached in animals. To study accumulation kinetics in the different fractions, three experiments were conducted with earthworms exposed to metal-spiked soil, and a natural polluted soil, and with indigenous earthworms exposed in field soils. Although kinetics showed variation, linear uptake and saturation type accumulation patterns could be understood using sub-cellular compartmentalization. For risk assessment purposes, sub-cellular distribution of metals may allow for a more precise estimate of effects than total body burden. Although more studies on compartmentalization kinetics and validation data are needed, the theory is shown to be promising, since it is physiologically understood and has a strong biochemical basis.

8.1 Introduction

Oligochaete species show a variety of accumulation patterns for trace metals, ranging from saturation-type accumulation curves to linear uptake curves in which elimination is approximately zero (Peijnenburg et al. 1999, Marinussen et al. 1997). This results in a wide range of body metal concentrations varying in biological significance. More specifically, the biological significance of a metal concentration depends upon the specific tissue in which the metal is deposited and toxicity cannot be predicted from total metal burden in the organism (Rainbow 2002). Sequestration mechanisms used by invertebrates to detoxify metals and prevent interaction with important biomolecules, include metal binding to proteins and other ligands, and storage in the inorganic matrix of granules. Using biochemical techniques, Wallace and Lopez (1996) developed a pragmatic method to quantify different metal sequestration forms based on several centrifugation steps. In this procedure, an organism is separated into a granular fraction, a tissue fragments and cell membrane fraction and a cytosolic fraction. The cytosolic fraction could be further separated into a microsomal fraction and heat-stable proteins (HSP) and heat-denaturated proteins (HDP). Due to the different metal affinity of the sequestration forms, it is likely that these metal fractions all have their own specific uptake and elimination kinetics. Understanding metal compartmentalization may explain accumulation and elimination patterns in organisms. Metals retrieved from fractions where they are loosely bound may easily be eliminated from the organism resulting in a saturation-type uptake curve, while metals bound tightly in inert fractions are eliminated slowly or not at all, resulting in linear uptake patterns. Differences in steady state levels in organisms can be ascribed to the additive results of accumulation kinetics of the different internal fractions.

The metal sequestration on a sub-cellular level has been summarized in a recently published review (Vijver et al. 2004), together with the possible consequences for accumulation patterns and toxicity. In general, it was seen that the granular fraction and protein fractions have the highest impact on metal uptake by fish and aquatic organisms. The fractions retrieved from lysosomes, nuclei, cell membranes, tissues, and intact cells were most important for elimination. In that way, these fractions were most important for the accumulation fashion, and the magnitude of steady state levels reached in organisms after exposure corresponds predominantly to the size of these fractions. Research on the distribution of metals over the internal fractions was up to now mainly performed with aquatic vertebrate (fish) and invertebrate species (shrimps, mussels, fresh-water oligochaetes). Studies on internal compartmentalization and its consequences for terrestrial organisms are scarce. As far as we know, only Conder et al (2002) and Honeycutt and co-workers (1995) separated soluble and pellet fractions inside earthworms using centrifugation techniques. These studies suggested that in the soluble cytosolic fraction, metal content increased in a linear manner during exposure, whereas in the pellet fraction consisting of granules, tissue fragments, and cell membranes, metals reached a steady state concentration. Dallinger and Prosi (1988) fractionated heavy metals from isopod hepatopancreases and demonstrated a metal-dependent distribution over pellet and supernatant.

Following exposure, organisms will invoke mechanisms to minimize interactions of metals with the receptor sites of target organs, at the same time ensuring sufficient delivery of essential metals to target organs. Differences in internal response may exist between laboratory-incubated organisms exposed to artificially metal-polluted soils and indigenous animals that are subjected to chronic exposure (Giguère et al. 2003). In this research, sub-cellular distribution of Ca, Cd, Cu, Pb, Zn, Mg, Fe, Ni and As was determined in terrestrial oligochaetes *Aporrectodea caliginosa* collected from the field. It was hypothesized that sub-cellular metal partitioning was metal-specific and that accumulation and elimination kinetics could be related to the binding of metals to certain sub-cellular fractions. To quantify the sub-cellular distribution kinetics of Cd and Zn over the different fractions mentioned above, metal concentrations in *A. caliginosa* were followed over time during exposure to Cd- or Zn-spiked soil. In addition an experiment was performed with *A. caliginosa* incubated in the laboratory exposed to a polluted field soil. To validate the laboratory observations and to investigate the differences in response between laboratory-incubated organisms and indigenous earthworms, indigenous earthworms were exposed to field soils, and after 61 days transferred into their soil of origin. Our motivation to transfer worms back into their soil of origin was that we assumed that the organisms would reach their initial concentrations after the total experimental duration of 118 days.

8.2 Materials and methods

Experimental design

Adult earthworms of the species *Aporrectodea caliginosa* were collected from several field sites and internal levels of Cd, Pb, Ni, Ca, Cu, Fe, Mg, Zn and As were determined aimed to obtain a general pattern of metal distribution over the different sub-cellular fractions. To investigate accumulation kinetics of the sub-cellular fractions three experiments were conducted.

Experiment 1: Adult earthworms of the species *A. caliginosa* were collected from a non-polluted forest soil in Lepelstraat, The Netherlands and kept in the laboratory for several weeks. To quantify metal kinetics of the three different sub-cellular fractions from which Cd and Zn were isolated, earthworms were exposed to spiked field soil and sampled at different time intervals; 4, 14, 21 and 28 days. Prior to the exposure of earthworms, field soil Epen, The Netherlands was spiked with a nominal concentration of 325 mg Cd/kg and 3100 mg Zn/kg, as metal acetate salts (purity 96%, Acros Chemicals). Ten weeks after spiking, DOC levels in pore water stabilised and this was used as an indication for the metal spike to be in pseudo-equilibrium with the soil.

Experiment 2: Adult earthworms of the species *A. caliginosa* were collected from a non-polluted sandy grassland soil in Liempde, The Netherlands and kept in the laboratory for several weeks. The earthworms were exposed to a field-polluted soil from Boxtel, The Netherlands. Earthworms were sampled at different time intervals; 0, 6, 13, 19, 28, and 35 days. Cd, Pb, Ni, Ca, Cu and Zn levels were analysed in the three sub-cellular compartments in the earthworms.

Experiment 3: Experiments using field soils and indigenous, mature earthworms were conducted to identify internal fractions from which the highest levels of metals could be isolated and to determine if initial Cd concentrations can be reached again. For this purpose, two bioassays were conducted with *A. caliginosa* collected from two different sites, namely a floodplain field soil ADW and the field soil Epen used in the spiking experiment 1. In these experiments, the worms from ADW were incubated in Epen soil and after 61 days transferred back into ADW for 57 days, and the reverse experiment was carried out as well. Earthworms were sampled at different time intervals; 0, 1, 3, 8, 13, 26, 42, 61, 63, 68, 92, and 118 days. Cd levels were determined in the five different sub-cellular compartments in the earthworms.

All experiments were conducted in glass jars (750 ml) with 400 g of moist soil and four earthworms in each jar. All soils were kept at a moisture content of 80 ±4 % of their maximum water holding capacity. After collecting the earthworms, they were placed on moist filter paper for 48 hours to void their gut contents and then frozen at -18°C. The experiments were performed under acclimatised conditions maintaining 80% relative humidity, 15±3°C and permanent illumination.

Prior to metal analyses, soil samples were dried at 40°C and sieved (<2 mm), followed by digestion in a concentrated HNO_3 solution (70% pro-analysed Baker) using a Mars5 destruction microwave oven. Total metal concentrations were analysed by ICP-MS (Perkin Elmer, SciEx ELAN 6000). Organic matter and carbonate content was determined by loss-on-ignition at 550°C and 900°C respectively. Pore water was collected using a permeable pore water sampler (Rhizon SMS-MOM, Rhizosphere Research Products, Wageningen, The Netherlands).

Fractionation of earthworms

Individual earthworms were thawed and homogenized using an Omni TH115 tissue homogenizer fitted with a 7-mm saw-tooth blade in 5 ml ice-cold 0.01M Tris-HCl buffer (pH 7.5, Fisher Scientific, Houston, TX). Homogenates were centrifuged at 10,000 g for 30 min at 5°C. Pellet fractions were boiled at 100°C for two minutes and hydrolysed at 60-70°C for one hour using 1 M NaOH (Merck, Darmstadt, Germany). The granules (fraction D) could be separated from tissue fragments, cell membranes and intact cells (fraction E) by centrifugation (MSE 16*10 rotor) at 10,000 g for 10 minutes. Initial supernatants, containing cytosol (C) were decanted, and the microsomal fraction (fraction F) was separated from the protein fraction by centrifugation (Phagus 10*10 rotor) at 100,000 g for 30 minutes. The protein fraction was heated for 10 minutes at 80 °C and placed on ice for one hour. The samples were then centrifuged at 30,000 g for 30 minutes to separate denaturated proteins (fraction G) from heat-stable proteins (fraction H). All supernatants were evaporated and all fractions were digested with HNO_3. For analyses, the dry residues were dissolved in 0.7 M HNO_3 (ultra-pure Sigma-Aldrich, Seelze, Germany). Metal quantification was performed on an atomic absorption spectrophotometer (Perkin Elmer 1100B) with flame and graphite furnace

capabilities and by ICP-MS (Perkin Elmer, SciEx ELAN 6000). Blanks were included and standard additions were within the range (15%), indicating good recovery in the matrix.

Calculations

Data on internal metal concentrations were modelled using a non-linear one-compartment model. Uptake rate constants and elimination rate constants of individual observations in time were fit with first order equation (eq. 1).

$$Q(t) = C_0 e^{-k_2 t} + \frac{a}{k_2} \cdot 1 - e^{-k_2 t} \qquad [1]$$

where Q(t) is the internal metal concentration in earthworms (µg g^{-1} wet weight) at time t (d), C_0 is the initial concentration in earthworms (µg g^{-1} wet weight), k_2 is the elimination rate constant (d^{-1}). a is the uptake flux, modelled as k_1 C_{extern} in which k_1 is the uptake rate constant (g_{soil} g_{animal}^{-1} d^{-1}) and C_{extern} is the metal concentration either in soil (mg kg^{-1}) or in pore water (µg L^{-1}). Each sub-cellular compartment was treated as an entity, because after metal entrance in the organism, immediately metals will be partitioned over the different sub-cellular fractions of which we assume the dynamics are independent of each other. Modelling and parameter quantification was done using Graphpad Prism ™ 2.0 (Graphpad Software, San Diego, CA, USA).

8.3 Results and discussion

Bio-classification of sub-cellular metal distribution

A qualitative generalized overall impression of Cd, Zn, Ca, Cu, Pb, Mg, Fe, Ni and As (by ICP-MS) distribution over the sub-cellular fractions in earthworms (n=138) collected from the field is given in Figure 1.

There is a metal-specific difference in the distribution over the internal fractions that can be explained from preferred biochemical binding and physiological use of the metals by animals. In general, essential metals are present at higher levels in the tissue fragment and cell membrane fraction (E), which can be attributed to their metabolical requirement. To buffer reactive free metal cations, many metabolic processes are known to sequester metals. The processes all play a role in the maintenance of essential metals. Reactive nonessential cations are also sequestered in order to decrease toxic stress (Rainbow 2002). The binding affinity does explain many of our findings on the sub-cellular distribution of metals in earthworms.

As seen in Figure 1, Ca, Mg and Zn could mainly be found in the tissue and cell membrane fraction (E). Zn occurs in mitochondria and in proteins (e.g. zinc fingers) and is distributed over the whole body. Ca is required for e.g. the structure of the exoskeleton, muscle contraction and blood clotting. Mg is a component essential to enzyme mediation (Avila 1995). Although As has no essential function, this metalloid was retrieved from the E fraction and a minor amount from the cytosolic fraction (C). Arsenate imitates phosphate, which explains the occurrence of As in the phospholipids that are part of the cell membrane. This is

in agreement with findings from the literature showing that As is mainly bound to S in glutathione (Langdon and Piearce 2003).

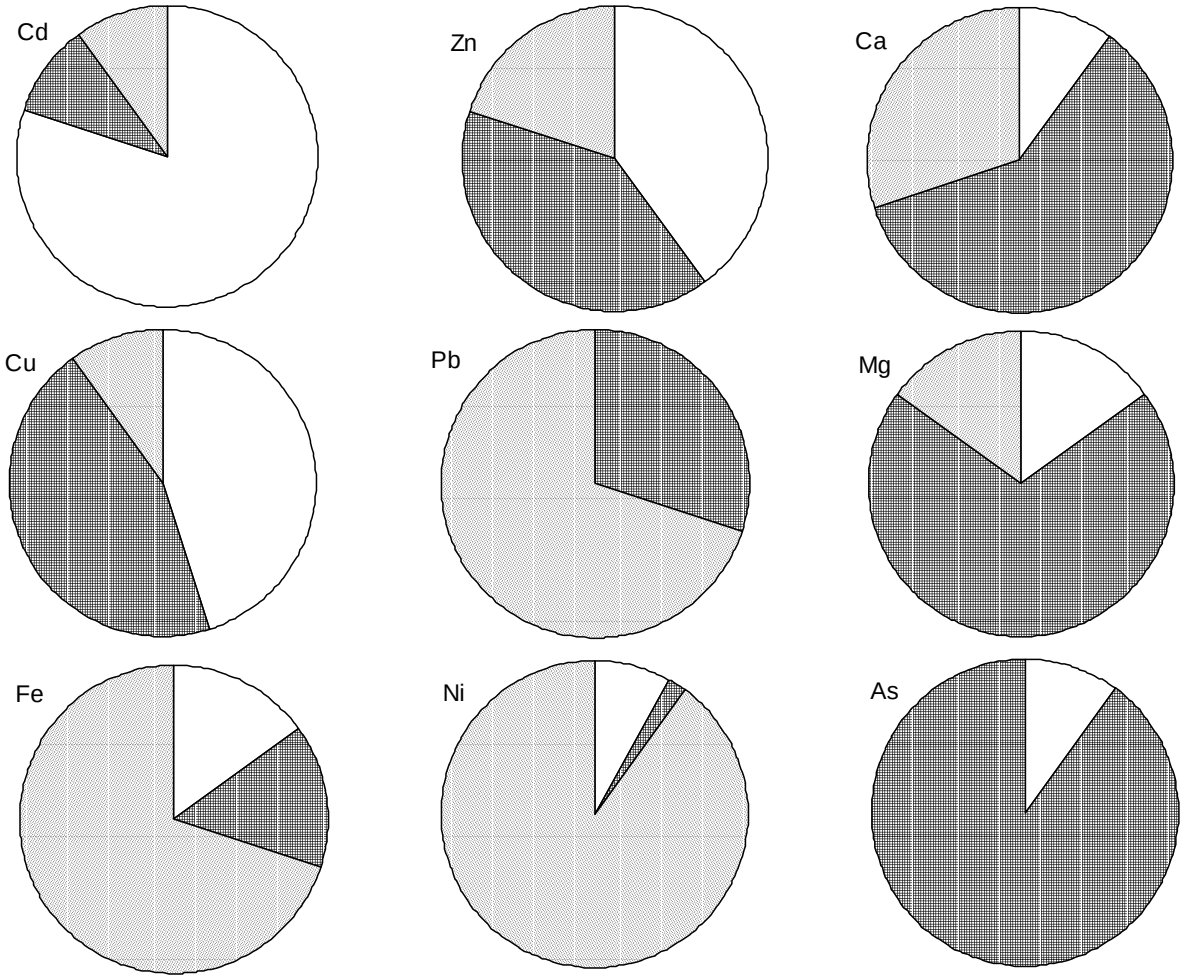

Figure 1: Overall patterns of metal distribution over the different fractions in the earthworm *Aporrectodea caliginosa* (n=138) collected from the field. D is the granular fraction (dotted), E is the tissue and membranes and cell organelle fraction (grid), C is the cytosolic fraction (white), including F, G and H fractions (= microsomal and HDP and HSP fractions).

Cu was equally distributed over the cytosolic fraction and the cell membrane and tissue fraction (E). The presence of Cu in the E fraction can be explained from its essential role in metabolism, its occurrence in the cytosolic fraction (C) from its role in the glutathion activities and superoxide dismutase to prevent organisms from adverse effects induced by reactive oxygen. Cu was bound to the same fractions in perch Giguère et al. (in prep). Contrary to Cu, Cd was mainly found in the cytosolic fraction (C) because of its binding to MT (metallothionein), which is induced to prevent Cd from competing with essential metals on the SH-sites of proteins and cystein. Cd, Zn, Ca, and Mg are partly found in the granular fraction consisting of type A granules (Hopkin et al. 1989) that are rich of calcium and phosphorus, Ca and P preferably bind these oxygen-seeking cations. The non-essential metals Pb and Ni were mainly found in the granular fraction (D) disposed with sulphur (present in

cysteine breakdown products), the so-called type B granules (Hopkin et al. 1989). Pb and Ni can also be packed in lysosomes originating from the endoplasmic reticulum (Avila 1995). Ni is known to have no clear binding preferences according to Nieboer and Richardson (1990), and therefore forms ligands with many functional groups in fish, for instance Ni was found in the microsomal fraction including lysosomes Giguère et al. in prep). Fe was found in the granular fraction (D), described by Hopkin et al. (1989) as type C granules consisting of ferritine, and in equal portions in the cytosolic fraction (C) and the tissue and cell membrane fraction (E). Fe is part of the transport protein ferritin that is concentrated in granules (for isopods known as Type C granule, Hopkin et al. 1989). Due to its essential role in the binding of oxygen in earthworms, Fe occurs in the cytosolic and metabolically required E fraction.
Similar to the chemically significant classification of metal ions in the environment (Nieboer and Richardson 1990, Hodson 2004), a classification of metal sequestration inside organisms is proposed here. It should be noted, however, that in case of sub-cellular distribution not only binding affinity to fractions (D-H) but also binding preference in certain organs and tissues (such as granules) needs to be accounted for. Subdivision of metal fractions as seen in Figure 1, was according to class 1 = Cd, class 2 = Cu, Zn, Ca, Mg, As, class 3 = Pb and Ni, class 4 = Fe, whereby the granular type which incorporates Fe is accounted. In this way, a pragmatic classification of metals over the sub-cellular fractions useful for screening purposes is formulated, from which the biological and toxicological significance of each fraction can be identified. This classification, however, does not give insight into the processes on a molecular level, but is widely applicable for all kinds of aquatic (Giguère et al. in prep, Wallace et al. 2003) and terrestrial organisms.

Soil properties
Actual total metal concentrations in the metal-spiked soil Epen were 582 mg Cd/kg dry soil and 4020 mg Zn/kg dry soil. Pore water metal concentrations in this soil were 1794 µg Cd/L and 10.29 mg Zn/L. Soil characteristics and metal concentrations in soil and pore water of the field soils are shown in Table 1. Keeping the soils at 80% of their WHC corresponded to a moisture content of 41.8 % w.w. for Epen (exp. 1 and 3), 39.0 % (w/w) for ADW (exp. 3), and 34.6 % for Boxtel (exp. 2).

Organism performance
No mortality of earthworms was observed in the bioassay (experiment 1) using Cd- and Zn-spiked field soil. Wet weight loss was negligible over 28 days of exposure, differences in wet weight before and after the experiment ranged between 17 and 27%. Earthworms exposed in experiment 2 too did not show mortality. Wet weights changed 38% over time. Mortality of earthworms exposed in field soils (experiment 3) was negligible and did not differ between treatments. Wet weights of *Aporrectodea caliginosa* did not change over the first 61 days of exposure. The average wet weight (mg) of earthworms sampled and sacrificed for metal analyses from the second half of the experiments were, after gut clearance: ADW-Epen-ADW 220 ± 130 (n=18) and Epen-ADW-Epen 255 ± 110 (n=16), which is 48% and 50% of initial

weights respectively. Most likely, differences in soil properties caused the change in wet weight of the earthworms.

Table 1: Physical/chemical characteristics and total and pore water (pw) metal concentrations of the field soils used in the three experimental designs. Dissolved organic carbon (DOC), the major anions and macro-elements Ca and Mg and cation concentration are given.

	[Ca] mg kg^{-1} d.w.	[Pb] mg kg^{-1} d.w	[Cd] mg kg^{-1} d.w	[Zn] mg kg^{-1} d.w	[Cu] mg kg^{-1} d.w	[Ni] mg kg^{-1} d.w	[Cr] mg kg^{-1} d.w	pH_{CaCl2}	LOI_1 %	LOI_2 %
Epen	2550	88.1	2.14	252	13.3	14.3	36.5	6.5	8.6	0.56
Boxtel	950	34.8	2.04	97.5	11.3	6.20	25.4	4.6	5.3	0.16
ADW	27850	127	3.65	476	57.2	35.9	95.8	6.8	6.7	-

	$[Ca]_{pw}$ mg L^{-1}	$[Mg]_{pw}$ mg L^{-1}	$[Pb]_{pw}$ µg L^{-1}	$[Cu]_{pw}$ µg L^{-1}	$[Cd]_{pw}$ µg L^{-1}	$[Zn]_{pw}$ µg L^{-1}	pH_{pw}	$[Cl^-]_{pw}$ µg L^{-1}	$[NO_4^-]_{pw}$ µg L^{-1}	$[SO_4^{2-}]_{pw}$ µg L^{-1}	DOC mg L^{-1}
Epen	71.4	16.5	2.43	7.56	7.35	1018	6.8	15	269	29.0	23.0
Boxtel	75.7	8.42	1.44	12.1	24.0	1838	5.1	47	170	113	19.2
ADW	181	9.41	0.32	21.1	1.82	72.0	7.2	25	325	60.5	25.0

LOI_1 is loss-on-ignition at 550°C (%OM), LOI_2 is loss-on-ignition at 900°C (%carbonate).

Exp. 1; Turnover kinetics of sub-cellular Cd and Zn in earthworms exposed to spiked soil

From the biochemical distribution of metals over the different fractions, elimination possibilities may be derived. *Aporrectodea caliginosa* were exposed for 28 days to field soil Epen, spiked with Cd-acetate and Zn-acetate. Initial total Cd concentrations in the earthworms were 4.08 ± 1.30 µg/g w.w. (n=11) and initial Zn levels were 164 ± 148 µg/g w.w. (n=10). During exposure of earthworms to the metal-spiked field soil, internal metal levels increased with time. The accumulation pattern of Cd and Zn over the different internal fractions, granular fraction (D), tissue, cell membrane and intact cell fraction (E) and cytosolic fraction including proteins and microsomal fraction (C), is shown in Figure 2. Total metal levels were calculated by summing metal concentrations of the sub-cellular fractions.

Accumulation kinetics were described over time using a one-compartment model (see equation 1) and uptake and elimination rate constants were quantified. Accumulation parameters and k_1 and k_2 values of the different sub-cellular fractions are given in Table 2, as well as the statistics of the model fit.

As can be seen from Figure 2, most Cd could be retrieved from the cytosolic fraction (C), which increased over exposure time. Cd in the cytosolic fraction (C) qualitatively contributes most to Cd accumulation, and also in quantitative sense the major part of Cd uptake kinetics (uptake flux = 4.44 g/g per day) could be explained from the cytosolic fraction (C). Cd levels in the tissue fragments and cell membrane fraction (E) also increased over time (influx = 2.51 mg/kg w.w.). Cd concentrations detected in the granular fraction (D) remained at a fixed concentration. The internal partitioning of Cd is in agreement with findings of Conder et al. (2002), who distinguished the cytosolic fraction (C) from the pellet fraction (defined as the D + E fractions in our experiment) in the earthworm *Eisenia fetida* exposed to Cd-spiked soil. In the case of *E. fetida,* the cytosolic fraction could be fitted to a linear regression model with a slope of approx 0.3. This linear increase of the cytosolic fraction could be explained by the extremely high Cd concentration spiked to artificial soil. The pellet fraction was modelled

according to a saturation-type one-compartment model (Conder et al. 2002). In both studies, and in the study of Honeycutt and co-workers (1995) it was seen that for Cd the cytosolic fraction (C) responds to the exposure concentration, whereas the pellet fraction (D + E fractions in our case) responds according to a saturation-type model.

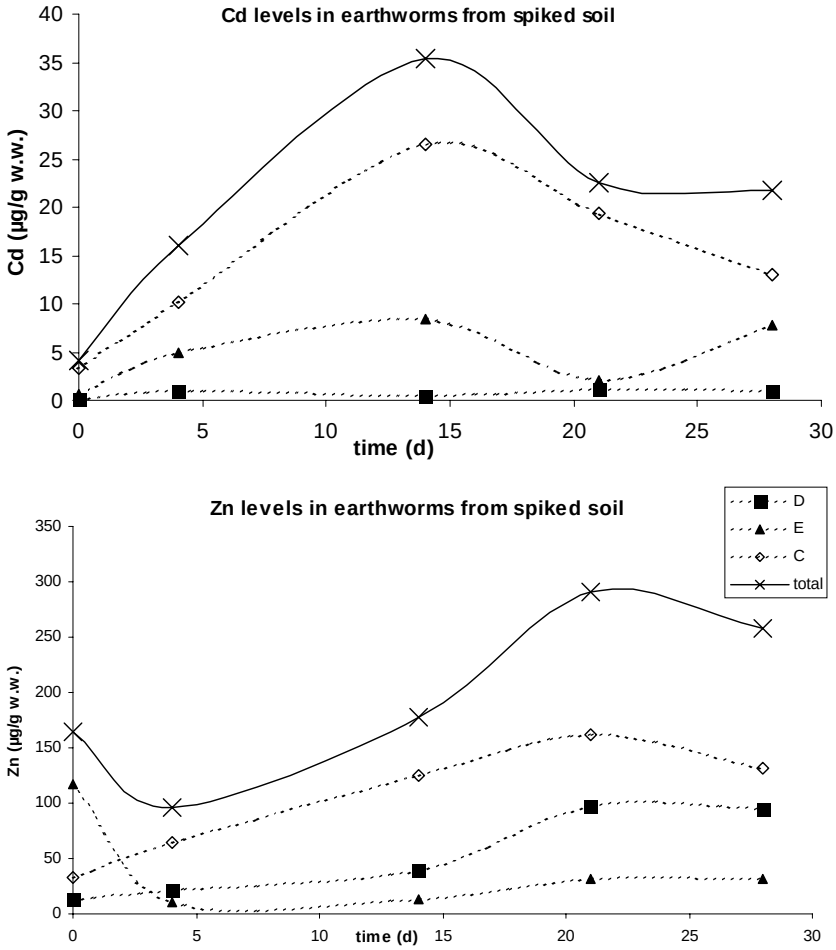

Figure 2: Sub-cellular distribution of Cd (upper) and Zn (below) in the earthworm *Aporrectodea caliginosa* during 28 days of exposure to a Cd-Zn (acetate) spiked field soil. D = granular fraction, E = tissue, cell membrane and intact cell fraction, C = the cytosolic fraction consisting of microsomal and protein fraction, total is the total body concentrations obtained by summing all sub-cellular fractions.

Zn concentrations showed a different response than Cd in the earthworms upon exposure to spiked soil (Figure 2). Zn could mainly be retrieved from the cytosolic (C) and granular (D) fractions, followed by the tissue, cell membrane and intact cell fraction (E). The latter fraction had a high initial concentration that more or less remained on a fixed level during exposure. Zn kinetics (Table 2) in the cytosolic fraction (C) were largest (both uptake and elimination, uptake flux = 16.7 µg/g w.w. per day and k_2 = 0.11 per day) compared to the kinetics of the D and E fraction. Zn in the granular fraction (D) had an elimination rate of zero. Comparing total accumulation kinetics of Cd and Zn showed that uptake rates do not differ much from each other, being approximately 7 µg Cd/g per day and 8 µg Zn/g per day, whereas the total elimination rate constant of Cd (0.27 per day) was larger compared to Zn (0.01 per day).

Table 2: Parameters estimated for uptake and elimination kinetics of Cd and Zn in the different sub-cellular fractions in *Aporrectodea caliginosa* over 28 days exposure to spiked field soil (eq 1). The uptake flux (a± se) is used to calculate the uptake rate constant k_1 ± se based on metal concentrations in pore water (ml $g_{animal}^{-1}d^{-1}$) and soil (g $g_{animal}^{-1}d^{-1}$), elimination rate constant k_2 ± se (d^{-1}), C_0 (± se) is the initial concentration (µg/g w.w.).

Cd	D*	E	C	total
C_0	0.73 ± 0.18	0.63 ± 3.52	2.60 ± 7.28	3.49 ± 8.11
k_2		0.42 ± 0.96	0.23 ± 0.33	0.27 ± 0.29
a		2.51 ± 5.50	4.44 ± 5.90	6.95 ± 7.16
k_1 pore water		1.40×10^{-3}	2.47×10^{-3}	3.87×10^{-3}
k_1 total		4.31×10^{-3}	7.63×10^{-3}	1.19×10^{-2}
R^2		0.47	0.66	0.74
Zn	D#	E*	C	total
C_0	10.1 ± 15.5	41.0 ± 19.7	29.1 ± 18.8	100.0 ± 52.3
k_2	0.00		0.11 ± 0.08	0.01 ± 0.08
a	2.91 ± 3.44		16.7 ± 9.43	8.41 ± 15.1
k_1 pore water	2.83×10^{-4}		1.62×10^{-3}	8.17×10^{-4}
k_1 total	7.23×10^{-4}		4.15×10^{-3}	2.09×10^{-3}
R^2	0.90		0.93	0.64

* = internal concentration in this fraction best modelled according to Q= constant, # = modelled best as a linear model in which k_2 is zero

Exp. 2; Sub-cellular distribution over time of Cd, Pb, Ni, Ca, Cu, Zn in earthworms exposed to field soil

Accumulation patterns in time in earthworms exposed to the polluted field soil Boxtel differed between metals (Figure 3) and could be described according to linear uptake in which elimination rates were zero, saturation-type curves and internal levels that did not change significantly during the whole exposure duration.

The sub-cellular distribution of Cd, Ni, Pb and Ca is exactly in line with the earlier findings in field worms (Figure 1). Cu was almost equally distributed over granular (D) and tissue fragments and cell membrane fractions (E) in earthworms exposed to field soil Boxtel, whereas the relative percentage of Cu in field worms in the granular fraction was lower. Nevertheless, the results are in line with each other, and the higher Cu uptake in the granular fraction can be ascribed to the relatively high Cu concentrations in the pore water together with the low pH in the Boxtel field soil (see Table 1).

Zn concentrations showed a slight difference in sub-cellular distribution among worms exposed to Boxtel soil (Figure 3) and worms collected from the field (Figure 1), which can be explained by the relatively low Zn body burden in worms exposed to Boxtel. The differences can be discussed conform the kinetic parameter estimates of each sub-cellular fraction. In Table 3 an overview is given of the accumulation types and estimated parameters for the granular fraction (D), the tissue fragments and cell membrane fraction (E) and the cytosolic fraction (C) for each metal species.

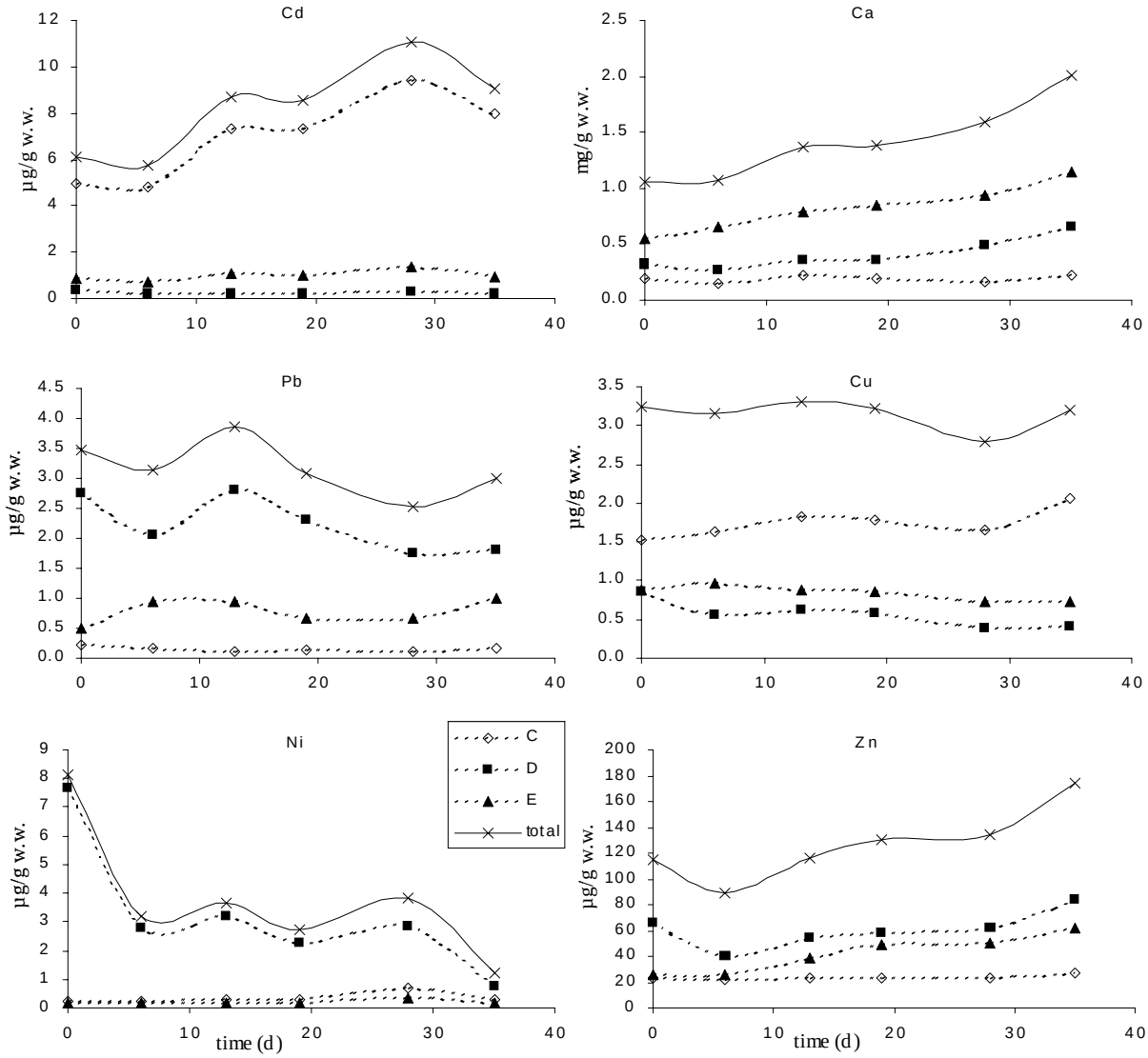

Figure 3: Sub-cellular distribution of Cd, Pb, Ni, Ca, Cu, Zn (µg/g w.w. except for Ca in mg/g w.w.) in the earthworm *Aporrectodea caliginosa* during 35 days of exposure to a polluted field soil Boxtel. D = granular fraction, E = tissue, cell membrane and intact cell fraction, C = the cytosolic fraction consisting of microsomal and protein fraction, total = the total body concentrations by summing all sub-cellular fractions.

Cd accumulation in earthworms exposed to Boxtel field soil and accumulation fashions of the sub-cellular fractions (Table 3) are in agreement with the findings on Cd accumulation in animals exposed to spiked Epen soil (Table 2). The only difference can be found in the amount of Cd retrieved from the tissue and cell membrane fraction (E) that remained on a fixed level in earthworms exposed to the Boxtel soil and showed a saturation-type curve in earthworms exposed to spiked soil. The difference of metal amounts in the E and C fraction between the two experimental series can again be explained from the exposure concentrations. The total (D+C+E fraction) accumulation pattern of Zn in earthworms exposed to Boxtel was described by linear uptake (Figure 3) whereas Zn accumulation of earthworms exposed to spiked Epen soil followed a saturation-type curve (Figure 2). When comparing the sub-cellular fractions, it can be seen that in both cases Zn in the E fraction varied between 45 and 55 µg Zn/g w.w. and the granular fraction (D) was in both cases equal to 75 µg/g w.w. As a

result, the only internal metal compartment in earthworms that varied between the two experimental series is the cytosolic fraction, which for earthworms exposed to spiked Epen soil contained up to 100 µg Zn/g w.w., and for animals in Boxtel soil 20 µg Zn/g w.w. Zn uptake in Boxtel is linear because the metabolic required Zn pool (reflected by the E fraction) and its buffer compartment (the granular fraction (D)) are both not on a level that is likely to cause overspill. Therefore, the cytosolic fraction is not yet initiated by the animal to sequester metals, which is different from the animals exposed to spiked Epen soil where exposure concentrations are higher compared to Boxtel soil and more internal detoxification is required to prevent the animal from adverse effects caused by reactive metals taken up. Total (D+C+E fraction) Zn concentration in earthworms exposed to spiked Epen soil was around 220 µg/g w.w., and for earthworms exposed to Boxtel field soil around 150 µg/g w.w.

Table 3: Type of accumulation pattern and the parameters estimated for Cd, Pb, Ni, Ca, Cu, Zn in the different sub-cellular fractions in *Aporrectodea caliginosa* over 35 days exposure to field soil Boxtel (eq 1). The uptake flux is a ± se (µg/g animal per day), elimination rate constant k_2 ± se (per day), C_0 ± se is the initial concentration (µg/g w.w.).

type of accumulation	metal species	fractions, kinetics and notes
Constant	Cd	D, E fraction
	Ca	C fraction
	Pb	C fraction
	Cu	D, E, C, total fraction
	Ni	E, C fraction
	Zn	C fraction
Saturation curve	Cd	C fraction, R^2 = 0.80
		C_0=4.54 ± 0.96, k_2=0.04 ± 0.06, a=0.43 ± 0.44
		total fraction, R^2=0.74
		C_0=5.6 ± 1.22, k_2=0.05 ± 0.07, a=0.51± 0.63
	Zn	D fraction, R^2=0.69
		C_0=53.8 ± 6.11, k_2=0.18 ± 0.20, a=9.64± 11.5
Linear uptake	Zn	E fraction, R^2 = 0.94
		C_0=24.3 ± 2.89, a=1.06 ± 0.14
		total fraction, R^2 = 0.73
		C_0=95.8 ± 11.6, a=1.83 ± 0.56
	Ca	D fraction, R^2 = 0.82
		C_0=0.24 ± 0.05, a=0.01 ± 0.002
		E fraction, R^2 = 0.98
		C_0=0.56 ± 0.03, a=0.02 ± 0.001
		total fraction, R^2 = 0.93
		C_0=0.98 ± 0.08, a=0.03 ± 0.004

D, E and total fraction of Pb and D and total fraction for Ni did not give a significant outcome in the models mentioned above.

In some cases, none of the accumulation models did fit the data. This happened for Pb and Ni, for which modelling with a non-fixed exposure concentration will likely give a significant fit. However, we did not measure exposure concentrations as a function of time.

Exp. 3; Sub-cellular distribution of Cd in indigenous earthworms exposed to soil
Accumulation results of earthworms originating from ADW exposed to Epen (until 61 days) and back into ADW (up to 118 days) and the reverse experiment of earthworms from Epen exposed to ADW and back into Epen soil, are depicted in Figure 4.

As seen in Figure 4, Cd levels in the different sub-cellular fractions showed a high degree of variation. Separation of the cytosolic fraction (C) into microsomal (F) and protein (G and H) fractions seemed to explain part of this variation. The Cd distribution over the different sub-cellular fractions was in agreement with the results found in animals in the experiments 1 and 2, where highest Cd levels were found in the cytosolic fraction (C). A general accumulation pattern in the indigenous earthworms (Figure 4) was hard to obtain. During exposure only slight changes may be seen. Remarkably, earthworms incubated in Epen and transferred back to ADW had elevated Cd concentrations in the second half of the experiment, especially reflected by the sub-cellular protein fractions HDP (G) and HSP (H). These same elevated internal Cd levels were not detected in the first half of the reverse experiment (Epen – ADW – Epen), which is basically the same experiment. This suggests that earthworms probably react in a physiological way (e.g. inducing stress proteins) to the change of incubation and exposure conditions.

Our expectation was that organisms after exposure to a certain field soil and transfer back in their soil of origin ultimately would reach their initial concentrations. This expectation holds for worms originating from Epen that had initial concentrations similar to internal Cd concentrations after 118 days of exposure, however, no significant accumulation was seen during the entire exposure period. Cd levels in earthworms originating from ADW did not return to initial concentrations. This indicates that stress of organisms may influence uptake and elimination kinetics, although no strong evidence was obtained from the experimental data (Figures 4). It might be concluded that the experimental design with reverse exposure regimes was too complicated. We dealt not only with many stress interferences together with metal stress, but also the use of indigenous earthworms caused a lot of scatter in accumulation data. One of the main reasons is wet weight of the earthworms at the start of experiments that already varied largely although all earthworms were mature. From the literature it is know that earthworms under stressful environmental conditions mature at lower weights to speed up the ability to reproduce (Klok et al. submitted).

A visible check for adulthood before the start of the experiment may therefore not be sufficient to reduce such variations in metal concentrations. In this stage of research the use of laboratory-incubated earthworms will shed more light on the biochemical and physiological mechanisms underlying sub-cellular distribution of metals in animals, and therefore be more suitable for validation of our sub-cellular distribution theory.

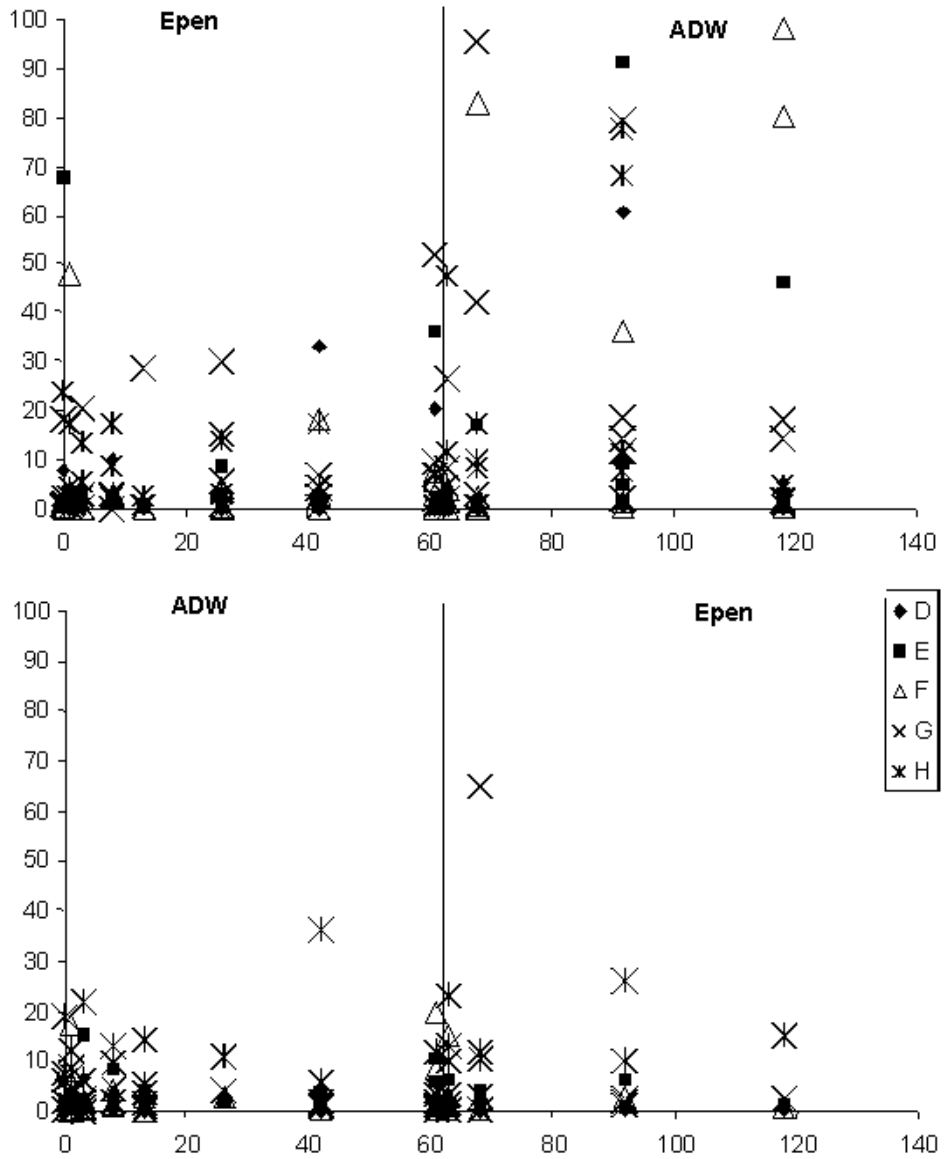

Figure 4: The sub-cellular distribution of Cd (µg/g w.w.) over time in the earthworm *Aporrectodea caliginosa* incubated in soil for 61 days and returned into its "own" soil for another 57 days together with the reverse experiment. D = granular fraction, E = tissue, cell membrane and intact cell fraction, F = microsomal fraction, G = HDP fraction, H = HSP fraction.

Implications for Environmental Risk Assessment (ERA)

To assess effects of chemicals on organisms, often Critical Body Residues (CBR) derived from laboratory experiments (McCarty and Mackay 1993, Lanno et al. 1998) are compared to metal accumulation levels measured in organisms from the field (Van Straalen 1996). One of the assumptions of the CBR concept is that metals accumulated in the organism are in equilibrium over the entire body. With this assumption, the capacity of organisms to sequester metals in forms that are not biologically reactive is neglected. Nevertheless, all detoxification mechanisms indicate that metal tolerance in invertebrates is realized by preventing metals from free circulation. Within organisms, many different ways of metal sequestration can be found. This biochemical sequestration of metals can be generalized over all organisms, e.g. earthworms in this study but also marine mussels, shrimps (Wallace et al. 2003), and fish

(Giguère et al. in prep). Bio-classification may be a fast indication of undisturbed metal distribution in organisms and can have an applicable pragmatic function in ERA.
To investigate which fraction can be associated with toxicity requires more research. For most metals in earthworms, granular and cytosolic fractions (see Figure 1) were responsible for metal immobilization and the metals in tissue fragments, cell membrane and intact cell fractions were mostly incorporated in the metabolic required pools. Knowledge on internal metal distribution in organisms may increase the predictive power of the CBR concept to estimate effect levels. The internal metal fractions that are in equilibrium with the reactive metal pool are responsible for the release of metals and the maintenance of a free metal pool, and thereby contribute to toxic pressure. In the same way as metal speciation in soil, the free reactive metal in the body that is likely to cause the adverse effects may only be a small fraction of the total metal body burden.

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Chapter 9

Relating metal accumulation to metal speciation in soil

Chapter 9
Relating metal bioaccumulation to metal speciation in soil

9.1 The research program

Effects of metal pollution are not always evident due to natural variations in metal concentrations in organisms over time. Effects on organisms from floodplain sites are even less evident due to the variation of environmental conditions, such as frequent inundation having an impact on soil properties and metal speciation, and the presence of vegetation. Therefore, Rijkswaterstaat (department RIZA) The Netherlands initiated a research program to derive a relationship between metal bioaccumulation and metal speciation in the soil, based on current scientific state-of-the-art knowledge.

This research program fits in the Dutch policy to enlarge natural areas and redevelop floodplains to increase the accessibility of water into floodplains. River redevelopment will cause several different environmental changes (e.g. change in water level, pH, redox conditions), which has further consequences for metal speciation in the system and for organisms. To enable judgement by decision makers, landowners and researchers of the ecological hazards caused by metals in redevelopment projects, research on metal problems requires integration of scientific results with management options. A start was made with the development of a pragmatic decision-support model (BIOCHEM), functioning as an assessment tool. The mechanistically based model was guided by two distinctive phases, namely a physico-chemical and an ecotoxicological investigation (Vink 1997).

The ecotoxicological investigation in this thesis deals with issues of metal bioavailability to soil invertebrates. The specific goals of this thesis were:

1. to relate metal uptake by soil invertebrates to metal speciation in soil
2. to relate metal uptake to the animal physiology of soil invertebrates, in particular of internal compartmentalization of metals
3. to derive a transfer function for metals from soil to soil invertebrates

This chapter incorporates knowledge on bioaccumulation mechanisms derived in this thesis in a more general overview of the work on metal bioavailability. Section 9.3 is written in the scope of the decision-support system BIOCHEM. An attempt is made to formulate transfer functions relating ecotoxicological modules to chemical modules. Validation of the model was performed using field data gathered during the research program.

9.2 Metal bioavailability: linking chemical availability with animal physiology

Metal bioavailability in Environmental Risk Assessment (ERA)

Variability in soil chemistry is not accounted for in current Dutch environmental quality standards, despite evidence that total metal concentrations in soil include nonbioavailable forms. Up to now, legislation is based on total concentrations in soil without considering any parameter influencing the mobility of metals. To enable a comparison between soils, for assessment purposes the total metal content is normalized with respect to organic matter and

clay contents (Vegter 1995). This normalization is based on natural background metal concentrations that differ among soils. Soil quality criteria should especially aim at distinguishing between soils that cause effects and soils that do not cause effects on biota. Organisms do, however, not assimilate total metal concentrations. Supplementary information along with total metal concentrations in soil is needed to predict metal speciation and to increase the reliability of predicting effects in the ecosystem.

Synthesis on the bioaccumulation mechanisms studied in this thesis

Uptake routes

For the assessment of metal bioaccumulation or toxic effects in organisms, the porewater hypothesis is often proposed to serve as a model (Van Gestel 1997), for which knowledge on metal partitioning in the soil over solid and soluble pools is required. A more refined assessment of bioaccumulation and risk of metals may be obtained by the Free Ion Activity Model (FIAM) (Campbell 1995) or Biotic Ligand Model (BLM) (Pagenkopf 1983, Morel 1986, Di Toro et al. 2001), whereby metal speciation in the exposure medium and modelling is based on free ion activity. The free metal ion is the metal species of interest for uptake over the biotic membrane (see Chapter 1, Figure 2). All the three approaches assume that the outer membrane of an organism is in direct contact with the external environment. Therefore, in the light of setting up a mechanistically based bioaccumulation or effect model for terrestrial organisms, it is very important to know the relative contribution of the dermal and oral uptake route (Figure 1). An essential part of the diet of earthworms consists of mineral soil particles (Doube et al. 1997) and decomposed matter (Piearce 1978).

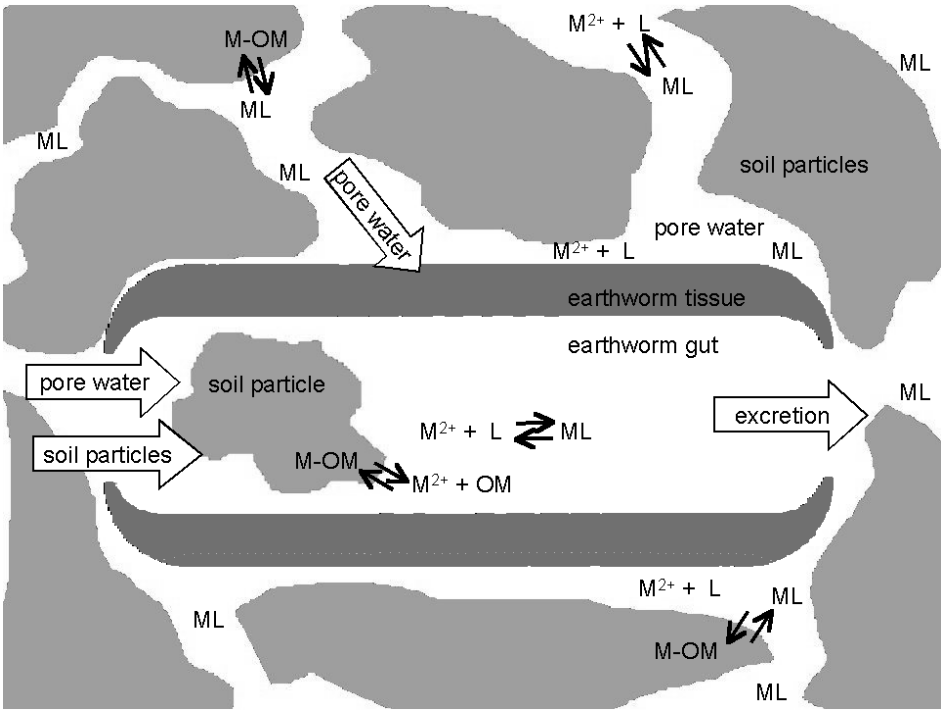

Figure 1: Schematic view of the uptake routes of chemicals in earthworms. The same principles hold for other soil-dwelling organisms. Metals taken up via the dermal route are influenced by metal speciation in soil; metals taken up via the oral route are influenced by metal speciation, which may be modified by gut conditions. For further details see text. M = metal, L = ligand, OM = organic matter.

Metals taken up via the dermal route can directly be related to pore water concentrations or specific chemical species in pore water. Metal uptake via the oral route cannot directly be explained from pore water concentrations or soil properties. Grinding ingested particles, the neutral pH of the digestive tract and the addition of digestive enzymes will affect the amount of sorption sites, which has impact on metal speciation (Edwards and Lofty 1972).
For terrestrial earthworms, the contribution of possible uptake routes is under debate. In this thesis the relative contributions of dermal and oral uptake routes were quantified (Chapter 3). Accumulation of metals was determined in orally blocked earthworms and compared to accumulation in non-blocked animals. It was found that the dermal route dominates over the oral route. The accumulation pattern up to 6 days for the different metals was from 70% up to 100% via the dermal route. From this result it was concluded that metals in equilibrium with the pore water are the source of interest. Uptake via the gut route has a contribution to bioaccumulation of metals, but the efficiency is much lower compared to the dermal route. The relative contribution of the dermal route might slightly alter when soil characteristics differ from the high pH and calcium-rich soils used in this study, although the dermal route will remain the dominant route of exposure. Under conditions in which the fresh versus dry weight ratio or the nature of biotic ligand changes, dermal uptake might get saturated, affecting the relative contribution. These aspects fall beyond the scope of the present research. The porewater hypothesis holds for terrestrial earthworms because the assumption of direct interaction between metal speciation and metal uptake is shown to be valid. Moreover development of a model, such as FIAM and BLM, known to work accurately in the aquatic ecosystem, also seems applicable to the terrestrial ecosystem.

Uptake kinetics

Uptake rate constants derived for the earthworm *Lumbricus rubellus* exposed to soil with and without extra food added did not differ significantly from each other (Chapter 4). This was expected since available evidence suggested that uptake of metals by earthworms occurs primarily from the soil rather than from food (Spurgeon and Hopkin 1999). Uptake rate constants (k_1) for Cd in experiments with and without food were 0.32 ± 0.07 and 0.22 ± 0.08 $\mu g_{soil}/g_{animal}/d$, respectively. This is consistent with the results in Chapter 3 describing earthworms exposed to the same floodplain soil. Here uptake kinetics (k_1) were estimated to be 0.26 ± 1.42 $\mu g_{soil}/g_{animal}/d$. For Zn the uptake rate constants in earthworms exposed to soil with and without food added were 0.42 ± 0.10 and 0.64 ± 0.10 $\mu g_{soil}/g_{animal}/d$ respectively, which were in line with the value of 0.36 ± 282 $\mu g_{soil}/g_{animal}/d$ obtained in Chapter 3. The use of uptake rate constants accounts for the chemical and biological availability of a metal.
Bioaccumulation kinetics were also derived for isopods exposed to radio-labelled soil or food or a combination of both (Chapter 5). Since these organisms have a firm cuticle, direct dermal uptake of metals from soil may be less important when compared to oral uptake (Van Straalen and Van Gestel 1993). Moreover, isopods are selective feeders, preferring organic matter from litter instead of organic matter from the mineral soil (Sutton 1972), so it was required to study explicitly the contribution of metals in the food. Rate constants for the uptake of Cd in isopods from soil or food were not significantly different from each other. The uptake rate

constants (k_1) in isopods exposed to ^{109}Cd-labelled soil was 0.038 ± 0.017 $\mu g_{soil}/g_{animal}/d$ and k_1 for isopods exposed to ^{109}Cd-labelled food was 0.014 ± 0.008 $\mu g_{food}/g_{animal}/d$. For Zn, the k_{1food} derived for animals exposed to ^{65}Zn-labelled food was lower (0.011 ± 0.002 $\mu g_{food}/g_{animal}/d$) compared to k_{1soil} for animals exposed to ^{65}Zn-labelled soil (0.033 ± 0.03 $\mu g_{soil}/g_{animal}/d$). The uptake rate constants from the different sources were shown to be additive. The quantity of metals taken up from soil or food appeared to be dependent on the exposure concentration.

Elimination kinetics

The steady state concentration in organisms is the net result of uptake and elimination. Organisms may control the elimination process, which therefore is not directly influenced by metals in the exposure medium and their bioavailability. Elimination has a large impact on the magnitude and time needed to reach steady-state concentrations. It was found that a precise estimate of elimination kinetics requires the involvement of physiological knowledge of internalised metals (Chapter 4). The internalised metals in the earthworm *L. rubellus* were divided over at least two types of compartments resulting in biphasic elimination kinetics. From the first compartment type, metals were able to eliminate in a relatively fast way, while the other compartment represented the metal fractions with slow elimination kinetics. Elimination kinetics of Cd and Zn from the fast compartment were ranging from 2.30 – 3.53 per day, and this compartment was emptied within approximately one to two days for Cd and two to three days for Zn. The storage compartment had a slow turnover; Cd was transferred from the first compartment to the storage with a rate constant of 0.08 per day and Zn with a rate constant of 0.008 per day. After 18 days of elimination, the animal still contained 46 % of the maximum amount Cd accumulated in 14 days of exposure. Only 7.5% of the maximum Zn concentration reached after 14 days of exposure was left in the body after the elimination period of 18 days. For comparison with elimination kinetics found in earthworms exposed in the same soil in Chapter 3, the k_2 values were recalculated by taking into account the duration of elimination. Average values of k_2 for Cd were 0.32 per day and for Zn 0.60 per day, which are in line with the elimination rate constants estimated in Chapter 3, namely for Cd 0.26 ± 0.13 per day and for Zn 0.81 ± 0.34 per day. The ability of earthworms to trap a portion of the metals into compartments is another argument for using estimations of bioavailability on the basis of fluxes.

Following uptake either from soil, food or both sources, metals in isopods moved towards a storage compartment (Chapter 5). Results on metal compartmentalisation and kinetics suggested that metals from the food move directly via the digestive tract into the hepatopancreas. Metals taken up from the soil remain longer in the digestive tract, to increase absorption possibilities of required elements, before they are translocated to the storage compartment. A large fraction of Cd is retrieved from the storage compartment, whereas Zn is mostly retrieved from the reactive compartment and therefore can be excreted more rapidly from the body. The storage fraction appeared to be inert for elimination of both Zn and Cd and the fraction of metals stored in this compartment was influenced by the uptake source. Most metal bioaccumulation could be explained from the internal compartmentalization of

metals in the isopod since elimination has a large influence on internal metal levels reached. This strong impact of the elimination parameter makes a direct prediction of bioaccumulation from chemical availability of metals in the environment difficult.

Comparison of accumulation between the two species

In Chapter 4 and 5, earthworms and isopods were exposed to the same floodplain soil enriched with a similar amount of radioisotopes. When comparing total accumulation of Cd, earthworms accumulate up to 17% more from soil than isopods. After 18 days of elimination, Cd was retained in the earthworm in higher amounts than in the isopod. For Zn, the uptake by earthworms was also higher compared to the isopod. However, after 18 days of elimination, 10% of the accumulated Zn was still retained in the earthworm, whereas the isopod eliminated only 60% of the accumulated Zn. Furthermore, it should be noted that food is a significant uptake source of metals for isopods, while for earthworms the contribution of this source is negligible (see above).

Adsorption and absorption on exoskeleton

In conventional bioassays determining metal accumulation, organisms are analysed directly after defaecation of their gut content, thereby taking the sum of externally adsorbed and internally absorbed metals (Langston and Spence 1995). From an ecotoxicological point of view, it is important to know if metals are adsorbed or absorbed, to have insight in the internal toxicological pressure. Absorbed metals harm the organism, whereas adsorbed metals on the outerside of the animal are toxicologically of less interest. Autoradiography pictures showed only little adsorption of Cd and Zn to the outer membrane of isopods and earthworms exposed for 14 days to radiolabelled soil and food (Chapter 6). Consequently, when focusing on effects of metal uptake on the organism itself, there is no need to correct for adsorption. Considering the negligible impact of adsorption on bioaccumulation, this parallels the description of the uptake process in aquatic organisms.

Internal metal compartmentalization

Internal metal distribution in invertebrate species was investigated and generalized, aimed to make statements on bioaccumulation strategies and to assess the toxicological metal pressure. As seen in Chapter 7, all organisms evolved mechanisms to control selective utilization of essential metals and that minimize damage of reactive forms of essential and nonessential metals. A diversity of specific metal accumulation strategies are known, however, distribution of metals over the different internal compartments is, on a biochemical basis, generally similar for different organisms. Two major types of sequestration are known to occur after increased exposure to metals. The first mechanism is formation of inclusion bodies (granules), the second is induction of proteins that have metal binding capacity. The way organisms deal with internalised metals is schematizised in Figure 2, modified from Rainbow (2002).

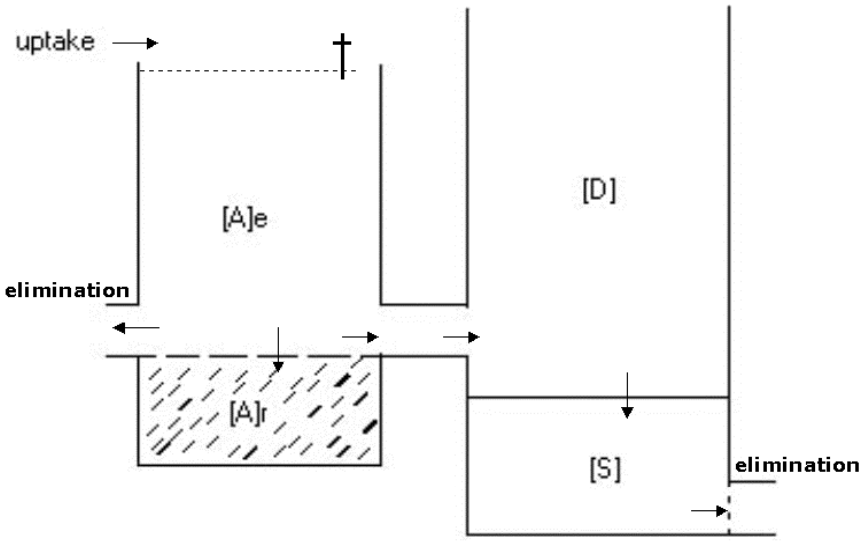

Figure 2: Generalized scheme showing the various compartments in which metals may be present and accumulate inside an animal (modified from Rainbow 2002). [A]r is metabolically required metal pool, for non-essential metals this pool is negligible; [A]e is excess pool above metabolic requirements, causing toxicity and eventually mortality when elimination or detoxification fluxes are slower than uptake rate; [S] is storage; [D] is detoxification.

For earthworms it is known that they are able to accumulate metals to a high degree. The ability to deal with high levels of accumulated metals can be ascribed to a slow turnover of tissues in which metals accumulate. Hence, for oligochaetes distribution of metals over different internal compartments was determined in an experimental set up, described in Chapter 8. The different internal metal compartments were isolated based on differences in biochemical binding affinity. Metal compartmentalization was shown to be metal-specific, which allowed deriving four classes of metals useful in identifying the biological significance of steady state levels reached in animals. Most essential metals, such as Ca, Cu, Mg and Zn were retrieved from the fraction consisting of tissue fragments, cell membranes and intact cells, which correspond to the metabolically required pool in the generalized scheme of metal distribution in an animal (Figure 2). The metalloid As was found predominantly in this fraction as well, probably because As strongly binds to phospholipids that are part of the cell membrane. All abovementioned metals can be subdivided in class 2 according to Chapter 8. Cd and again high amounts of Cu were mainly retrieved from protein fractions induced under metal-stress conditions by the earthworm itself (class 1 according to Chapter 8). The protein fraction corresponds with the detoxification pool in Figure 2. Pb, Ni and Fe were mainly found in granules having high metal-binding capacity. Pb and Ni could be isolated mainly from the type B granules (the storage pool in Figure 2) and were identified as class 3. Fe could mainly be retrieved from type C granules (also depicted as storage pool in Figure 2), which partly showed to be in contact with the excess metal pool [A]e resulting in exchange abilities between compartments. Therefore, Fe was transferred into class 4 according to Chapter 8. Bio-classification may be a fast and pragmatic indication for environmental risk assessment (ERA) purposes for identifying undisturbed metal distribution within organisms.

Although uptake and elimination kinetics of metals over five distinctive compartments could only be quantified with a large uncertainty, it gave a general idea on multi-phase kinetics in organisms. Moreover, a new light was shed on effects assessment based on Critical Body Residues (CBR). It is clear that internal body concentrations more directly reflect the intrinsic activity of a metal compared to external metal concentrations. Nevertheless, CBRs tend to loose predictive power when toxicologically available and non-available fractions are present within the organism. After all, the CBR approach assumes that organisms will die at a fixed total internal concentration. Metal distribution is considered to be at equilibrium over all body compartments. One vital organ will ultimately fail and this will cause death. Models used to predict metal toxicity to organisms, other than fish, are based upon metal speciation in solutions and use the theoretical basis of the CBR approach. Nevertheless, results in Chapters 7 and 8 showed organisms having a non-equilibrium distribution of metals over various tissues and organs. At the cellular level, organisms have evolved control mechanisms to minimize accumulation of reactive metal species. Storage of internalised metals in granules and binding to proteins are two major types of cellular sequestration found in organisms. This internal sequestration caused that effects occurring within organisms were not proportional to metal binding to the outer membrane, which is the main assumption of BLM.
The CBR or BLM does work when organisms take up metals predominantly via the epithelium of respiratory organs. In general, these organs have very efficient uptake ability, even more efficient than uptake via gut epithelium (Van Straalen and Verkleij 1991). Furthermore, respiratory organs are the first target sites, and therefore in fish overall effects often are proportional to effects on the gills, because a direct relationship exists with osmotic pressure, blood composition and gas exchange (Pagenkopf 1983). Another example for which the CBR approach still holds are small organisms quickly achieving an internal equilibrium for metals due to a large ratio between body surface and content. For macrofauna having internal metal compartmentalization, finding an indicator fraction proportional to the occurrence of effects is deemed necessary for essential and non-essential metals. The fractionation procedure outlined in Chapter 7 may be a first step towards a practical tool to identify a fraction explaining most of the variability observed in metal accumulation and toxicity in organisms. Although identification of the fraction being most predictive for stress appeared to be difficult in our experimental set up (Chapter 8), the partitioning of metals to the fractions (cytosolic and granular fractions) in exchange contact with the metabolic required fraction (tissue fragments, cell membrane and intact cell fraction) are indicative of early toxicological pressure (Chapter 7). Categorization of effects according to their associated target site is therefore the next step for setting up predictive models across different species.

Recommendations on bioavailability and bioaccumulation

From this thesis, recommendations for data interpretation in field monitoring and laboratory studies can be extracted concerning metal uptake and toxico-kinetics of metals in soil invertebrates.

Simultaneous consideration of bioaccumulation and exposure dynamics

Simultaneously to bioaccumulation experiments, metal speciation in the solid phase and solution needs to be measured. This way the role of metal species in soil in bio-uptake can be evaluated separately. Net accumulation of Fe, Ni, Cu, Zn, Cd, and Pb in the earthworms was found to be at least two times higher than the absolute amount of metals present in soil pore water, except for Ca (Chapter 4). Apparently, replenishment of pore water metal concentrations by desorption of metals from soil particles is important and positively influences metal uptake. Moreover in experiments of Van Gestel and Koolhaas (2004) it was concluded that Cd desorption from soils was a fairly slow process, expected to take in fact more than several hours. Vink (2002) measured local depletion and collected sediment-dwelling organisms from the same sediment layers concomitantly. Even local disruptions in the free metal ion concentration were reflected by accumulation patterns over time. This illustrated that knowledge on free metal ion concentrations and metal species in direct relation with this pool, is required for understanding accumulation kinetics and/or modeling based on FIAM and BLM.

Some pilot experiments on metal bioaccumulation by earthworms exposed in an inert sand matrix flushed with metal-contaminated water and an added chelating agent Chelex were conducted (not published in this thesis). Chelex eliminated free metal ions. Using this set up, the impact of free ion activity versus total dissolved metal concentration on uptake by earthworms might be quantified. Although we did not study this in detail, the pilot study suggested that replenishment of metals towards Me^{2+} and fast dissociating Me-Ligands (such as OH) can be rate limiting. In another pilot study (not published in this thesis), evaporation of pore water from the soil caused a change in metal equilibrium towards the solid phase, resulting in an elimination flux of radioactive metal from the earthworms.

The impact of free ion concentrations on bioaccumulation is relatively small when the exposure medium is essentially infinite, because the concentration of potentially free metal ions remains constant by the large bulk concentration of loosely bound metals (Pinheiro et al. 2004). Nevertheless, metal partitioning over different soil phases may change with time (Sauvé et al. 2000). Especially with high biomass or low available concentrations, local depletion may always occur. As metal bioavailability in soil is a dynamic entity, it should be realized that single status measurements are not sufficient to get insight into bioaccumulation. Moreover, exposure (chemical) sampling should be extended by some external compartments describing chemical release kinetics towards the pore water of metals bound to soil solids.

Accumulation modelling to obtain turnover kinetics, and especially elimination kinetics, is most precise when steady-state levels in organisms are reached. It should be taken into account that many organisms have accumulative tissues that have a very slow turnover rate, and hence, experiments need to last for a long time. The initial uptake rates can best be assessed when having many measurements in the first days of exposure. Summarized, for accurate bioaccumulation measurements sampling in time is required. As seen before, bioavailability and bioaccumulation are both dynamic processes that best can be described using fluxes over time.

Sub-cellular dynamics

It was seen that sub-cellular compartment modelling refined bioaccumulation and effect assessment on the individual level. For earthworms, five different sub-cellular compartments could be identified, all having their own metal-binding affinity. The quantification of metal uptake and elimination kinetics of each compartment needs some refinement before strict conclusions can be drawn. Experiments performed in a well-known matrix, such as OECD artificial soil or LUFA2.2 soil, spiked with metal-acetate salts, are proposed to study the mechanisms of the metal accumulation pattern of each fraction.

In the kinetics modelling of the sub-cellular compartments the starting-point was that all five fractions are able to take up metals from the environment and eliminate individually independent from each other. The compartments therefore were seen as anatomic entities. This way of modelling was chosen because after entrance in the organism, metals will immediately be partitioned over the different sub-cellular fractions of which the dynamics are independent of each other. It is recommended to test this starting point and determine whether some compartments are fed by other compartments and with what type of dynamics.

Another point in the internal metal distribution modelling requiring some detailed research is whether uptake routes of metals have an impact on the internal circulation of metals through the body and hence, on the transient metal binding. It is not unlikely that this temperate binding of metals before incorporation into an inert compartment results in another internal fractionation, dependent on the dermal or oral uptake route. The final compartmentalization may even differ between uptake routes, and the duration of exposure may influence metal fate. These aspects require some more mechanistic research.

Summarizing, it can be concluded that chemical metal availability in the soil and uptake and subsequent accumulation over the various sub-cellular compartments are dynamic processes. To understand the complex concept of metal bioavailability and the resulting bioaccumulation in animals, the focus should be on the dynamics of metal movement between and along all compartments. A more complete picture of bioavailability and bioaccumulation containing the processes discussed in this thesis is depicted in Figure 3.

Metals are partitioned over solid and solution phases in the soil. Metals can be exchanged between the compartments. The metal species most likely to be taken up by organisms is the free metal ion. Although internal compartmentalization is modelled as anatomic entities, based on the knowledge synthesized from the literature (Chapter 7) and the experiments in Chapter 4, it is more likely that the compartments do have interactions with each other. As mentioned before, the tissue fragments and cell membrane fraction (E) represent the metabolic required metal pool, which is protected from overspill of reactive cations by elimination and exchange abilities to the cytosolic fraction (C) that can induced by the organism itself. Metals bound to proteins can be transported to excretion organs and/or to detoxification fractions, such as granules (D). Granules are known to have exchange contact with the metabolic required pool (E) as they have a buffer function for essential metals.

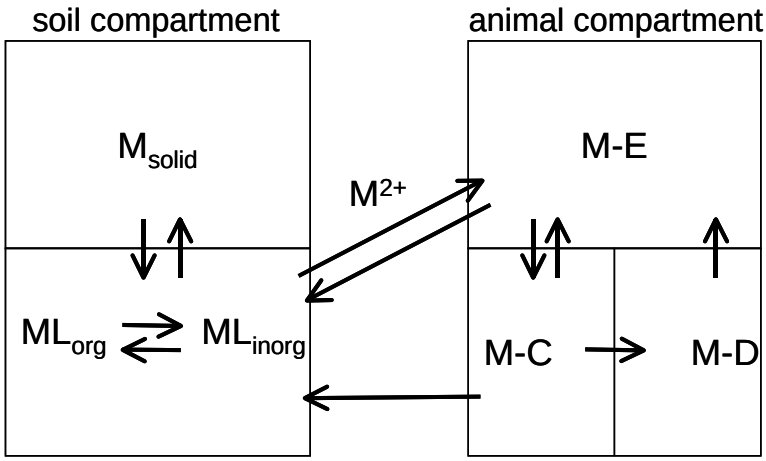

Figure 3: Generalized scheme of the dynamics of bioavailability, bioaccumulation and sub-cellular compartmentalization of metals in soil invertebrates. M = metal, L = ligand, org = organic and inorg = inorganic. The internal compartments are defined as in Chapter 8. D = granular fraction, E = tissue fragments, cell membrane and intact cell fraction, C = cytosolic fraction; C can be subdivided into F = microsomal fraction, G = heat-denaturated protein fraction and H = heat-stable protein fraction. The arrows represent metal fluxes, in more detail explained in text. A food compartment can be considered in the same way as the soil compartment.

This general scheme of the dynamics of bioavailability, bioaccumulation and sub-cellular compartmentalization of metals can be quantified in form of a case-study BIOCHEM. Scenario analysis was performed for metal transfer in organisms inhabiting floodplain systems as described in Chapter 2.

9.3 Case-study BIOCHEM: Pragmatic metal transfer functions for use in Environmental Risk Assessment

For the purposes of ecological risk assessment, there is a need to develop a model that will predict the bioaccumulation and toxicity of metals to soil invertebrates, that is not only based on the abiotic modifying factors of bioavailability, but also on the internal mechanisms that detoxify bioavailable metals. As mentioned before, the mechanistically based model BIOCHEM accounts for physico-chemical and ecotoxicological parameters.

Input and output parameters of the chemical module

With the chemical modules of the decision-support model BIOCHEM (Schröder and Vink, pers comm.) changes in metal concentration and speciation in soil over time and depth can be calculated. The model gives an estimate of metal speciation in relation to soil properties. Since processes such as adsorption/desorption and precipitation/dissolution, and other buffering phases are accounted for, it is possible to assess metal speciation under scenarios of fluctuating environmental conditions. The model input of the chemical module is total metal concentrations. Total metal concentrations are chosen because they are often reported in the literature, after all, current risk assessment is based on total metal levels in soil. Additionally, measurements of total concentrations are easy and cheap to obtain. Manipulating input parameters of soil properties can predict oscillations in speciation due to inundation of floodplains and/or change of land use. The output of the chemical modules explicitly specifies different speciation forms. The different metal species are classified according to three

categories, total metal concentration (including an inert part), a sorbed fraction and a dissolved fraction in pore water. The latter fraction can be sub-divided into a metal pool bound to organic acids (DOC), bound to an inorganic fraction (Cl^-, OH^- etc.) and the free ion activity. In this way, the model provides a simplification of the actual situation into modules, which accurately assess the distribution of metals among the soil and solution phases.
This fully parameterized geochemical model can be used to explain location-specific differences and especially whenever redox conditions change over time. However, to explain field data over a wide range of soils in general terms, equilibrium conditions are often assumed. The fully parameterised geochemical model can be simplified without losing predictive power (Schröder et al submitted). The simplified model presents the metal concentration in the pore water as a function of the total metal concentration in the soil, the total Ca concentration in the soil (including calcite, which is important in Dutch floodplain soils) and the particular organic carbon and clay content. Metal levels in the pore water assessed by the model were verified by metal concentrations obtained after 2.5 mM $CaCl_2$ extraction, with molarity of the extract reflecting ionic strength of the soil solution (average value for Dutch floodplain soils).

Output of the chemical module linked to the ecotoxicological module
To link metal speciation to bioaccumulation, two types of transfer functions are proposed. These two transfer functions are based on differences in uptake routes in the organisms. The first function is written for a group consisting of organisms having a highly permeable cuticle, such as earthworms. The second group involves species having a firm exoskeleton, such as isopods that have a chitin hardened calcium-rich carapax (epidermis).
Bioaccumulation models are constructed using uptake and elimination kinetic parameters, in this way taking into account that in- and out- fluxes are distinctive processes due to various mechanisms like internal compartmentalisation. For both animal groups it was seen that simply using one of the metal species as input parameter is insufficient to cover all aspects of bioaccumulation. For that reason, two output parameters of the chemical module were used as input parameters for the transfer function describing bioaccumulation in organisms.

Transfer function 1
For earthworms and related species, uptake and elimination can be described as a two-compartment model with first order kinetics. During exposure of earthworms to a polluted soil, accumulation in time (Cw(t) in µg/g) can be estimated according to equation 1 describing the first compartment and equation 2 describing the second compartment of the bioaccumulation.

$$Cw(t) = \frac{a}{k_2 + k_i}\left[1 - e^{-(k_2 + k_i)t}\right] \qquad [1]$$

$$Cw(t) = \frac{k_i \cdot a}{(k_2 + k_i)^2}\left[(k_2 + k_i)t + e^{-(k_2 + k_i)t} - 1\right] \qquad [2]$$

Equation 1 and 2 merged together gives:

$$Cw(t) = \frac{a}{k_2 + k_i}\left[1 - e^{-(k_2+k_i)t}\right] + \frac{k_i \cdot a}{(k_2 + k_i)^2}\left[(k_2 + k_i)t + e^{-(k_2+k_i)t} - 1\right] \quad [3]$$

in which k_2 is the elimination rate constant (d^{-1}) (derived in Chapter 4), k_i is the rate constant for internal metal transfer from the loosely-bound metal compartment towards the storage compartment (d^{-1}) (derived in Chapter 4). t is time in days. The parameter a represents metal uptake flux (= $k_1 \cdot$ external metal concentration) separated in two fluxes either via the dermal or oral route, defined as:

$$a = k_1 d \cdot [Me]_l + k_1 o \cdot [Me]_x \quad [4]$$

in which the first metal species $[Me]_l$ is representing the total pore water metal concentration (µg/L). Output parameter from the chemical module is metal concentration (in µg/L) determined using 2.5 mM $CaCl_2$ extraction. The second metal species $[Me]_x$ represents metals bound to inorganic and organic ligands and bound to organic matter and can be taken up under earthworm's gut conditions. In the chemical module total metal concentrations (in mg/kg) in the soil are measured by the metal concentration obtained using 0.43 M HNO_3 extraction.

Uptake rate constants k_1 are separated into k_1d and k_1o, and cannot be lumped together. k_{1d} is defined as uptake rate constant of the dermal route ($ml/g_{animal}/day$) based on total pore water concentrations and k_1o is the uptake rate constant via the oral route ($g/g_{animal}/day$) based on total metal concentrations. To account for the efficiency of metal uptake in relation to e.g. food or soil ingestion, soil type is incorporated in the uptake rate constants. For the modelling, the distribution of metals over the sub-cellular fractions was simply divided into four groups of bio-classification (Chapter 8). As seen in Chapter 4 and 8, metal partitioning over the different sub-cellular fractions has an impact on the elimination rate kinetics. Kinetic values and the relative contribution of the dermal and oral uptake route for each metal are given in Table 2.

The fraction of metal taken up via the dermal route is denoted D (derived in Chapter 3 and 4), and (1-D) is the fraction of metals available for uptake through the oral route. This relative contribution is the product of the uptake rate constants via the dermal and oral route and can be used as a check on the calculation of metal bioaccumulation in earthworms. The k_1 based on a labile radioisotope pool and k_2 values for Cd and Zn were measured in Chapter 4. Elimination kinetics k_2 of the other metals are calculated as a weighted average from data of Chapter 3 with the knowledge on internal fractionation derived for Cd and Zn in Chapter 4 and 8. The k_1d and k_1o values are estimated from total metal influx divided into an oral influx and a dermal influx. The values reported in Table 2 are in agreement with the measured values. The k_1d for Cd (Chapter 4) was 1.27 $ml/g_{animal}/day$ and k_1o 0.0005 $g/g_{animal}/day$, the

k_1d for Zn (Chapter 4) was 7.3 ml/g_{animal}/day and k_1o 0.00006 g/g_{animal}/day. The external metal pool on which the values are based can explain differences in the uptake rate constants.

Table 2: Accumulation kinetics and D values (% uptake across the dermal route) for transfer function 1. The k_1d and k_1o values are estimated from data belonging to Chapter 3 and unpublished data (flux oral = total influx – flux dermal), whereby the flux = k1 Ce. The ki values for all metals were derived from Cd and Zn and the internal distribution as described in Chapter 8, k_2 was calculated as a weighted average.

metal	bio-class	D (%)	k_{1d}	k_{1o}	k_2	k_i
Cd	cytosol	83	1.39	0.004	3.10	0.08
Zn	tissue	70	7.77	0.003	3.53	0.008
Cu	cytosol/tissue	100	1.12	0.0005	3.10	0.01
Pb	granules	100	67.6	0.0003	6.33	0.008
Ni	granules	84	1.33	0.01	> 0	0.008
Ca	tissue	87	0.45	#	3.53	0.01
Fe	ferritin granules	96	236	0.02	> 0	0.08
Mg	tissue	75*	n.d.	n.d.	n.d.	n.d.
As	tissue	50*	0.02	0.03	> 0	0.008

* = assessed using knowledge of the other metals having similar action. # = estimated to be negative, resulted from mass-balance calculation. n.d. = not determined.

After transfer of earthworms from a polluted to a clean soil, internal metal concentrations in time (Cw(t)) can be estimated according to equation 6. The metal pool in the soil decisive for defining a soil more or less polluted is the soluble metal concentration, because bioaccumulation predominantly can be described via the dermal uptake route.

$$Cw(t) = eq1(Te) \cdot e^{-(k_2+k_i)(t-Te)} + \frac{k_i eq1(Te)}{(k_2+k_i)}\left[1-\exp^{-(k_2+k_i)(t-Te)}\right] + eq2(Te) \quad [6]$$

in which eq1 = equation 1, and eq2 = equation 2. Te is the time over which earthworms were exposed to the polluted soil.

Transfer function 2

For organisms having a firm exoskeleton, uptake and elimination can be described as:

$$Ci(t) = \frac{a}{k_2}\left[1-e^{-k_2 t}\right] \cdot \left[Fi + (1-Fi)e^{-k_2(t)}\right] \quad [7]$$

in which k_2 is the elimination rate constant (d^{-1}), t is time per day. Fi is the fraction (ranging from 0 - 1) that cannot be eliminated and is stored in the body. a is defined as the uptake rate flux, separated into a pool of metals taken up via soil and a pool taken up via food. Metal accumulation in isopods is for 50% from soil and 50% from food intake (Chapter 5).

$$a = k_{1s}[Me]_x + k_{1f}[Me]_p \quad [8]$$

whereby k_{1s} is the uptake rate constant from the soil (in $g/g_{animal}/day$) and k_{1f} is the uptake rate constant from the food (in $g/g_{animal}/day$). The metal species $[Me]_x$ represents the total metal concentration in soil (including metals bound to inorganic and organic ligands and bound to organic matter). Because litter and food are not accounted for in the chemical module, it is proposed to model the input for the transfer function via the plant modules, which have also been developed within the BIOCHEM project (Schröder and Vink, pers commun.). The metal species $[Me]_p$ represents the metal concentration in litter, which for this purpose is equalled to the output from the plant module multiplied by a factor of 10.
The schematic model-route is chemical module → plant module → animal (firm cuticle)
The plant module consists of three types of models; for monocot, dicot (wood and herbs) and Leguminosae (nodule formers fixing nitrogen by means of *Rhizobium* bacteria). It is assumed that isopods mainly feed on decomposed material of the group of dicotyledonous plants. Metal accumulation in plants differed from metal levels found in plant material subjected to decay (due to leaching, weathering, fragmentation). Although metal-specific, metal levels increase when plants become litter. Cd and Cu levels increase with a factor 5, Cu and Zn with a factor of 15, Pb with a factor 55, Fe with a factor 80. The concentration factor is set at a value of 10, which agrees with Ca concentration differences between fresh leaves and litter in a deciduous forest (Froment et al 1969).
The uptake route has different impacts on the uptake kinetics, elimination kinetics and internal metal compartmentalization. Accumulation kinetic parameters of Cd and Zn for isopods are given in Table 3.

Table 3: Accumulation kinetic parameters for metals in isopods according to transfer function 2. Kinetic parameters and stored fraction (Fi) are derived from Chapter 5

metal	parameter	Soil	Food
Cd	Fi	0.43	0.64
	k_1	0.02	0.02
	k_2	0.19	0.18
Zn	Fi	0.55	0.61
	k_1	0.02	0.02
	k_2	0.18	0.23

Validity of parameters in different soils
In case earthworms or isopods are transferred into a soil with characteristics different from floodplain soils, extrapolation of the uptake and elimination rate constants is required. In these cases the transfer functions are semi-mechanistic and indicative for the maximal potential uptake, thereby intended to estimate the internal metal concentrations in organisms influenced by dynamic external concentrations in time. For earthworms, the k_1d that is connected with soluble metal concentration can alter with differences in soluble metal species. A larger impact on bioaccumulation modelling is expected for k_1o that is connected with metal concentrations obtained using 0.43 M HNO_3 extraction. Soil characteristics do not only influence the distribution of metal species over the total metal fraction, but also have consequences for feeding rates. For isopods the same reasoning holds, but here the parameter

with the highest uncertainty for extrapolation is k_{1s}. The parameter k_{1f} is assumed to be quite stable, because the food preference of isopods is species-specific and less soil dependent.

Using transfer function in the dynamic field situation

Accumulation kinetics were derived in experiments under laboratory conditions. The exposure duration of 28 days was not sufficient to reach steady state levels. Spurgeon and Hopkin (1999) and Sheppard et al. (1997) did not find steady state levels for Cd and Zn in earthworms after 50 days of exposure. To estimate accumulation kinetics of animals exposed in floodplain systems, knowledge on the exposure time is needed. This especially counts for assessing the second (storage) compartment in earthworms (eq2) and the inert fraction in isopods (eq8), which in some cases mathematically show continuous accumulation. Nevertheless, the exact exposure duration of animals in the field is hard to determine. Klok et al. (submitted) showed that the life cycle of the earthworm *L. rubellus* inhabiting dynamic floodplain system was adapted to the frequency of flooding events. For this reason it is assumed that the period in between flooding represents the exposure duration. The period between flooding differed each year (see Chapter 2, Figure 2) and averaged around 6 months. For the modelling, an average exposure time of 140 days was assumed with just after flooding a life duration of 56 days (no juveniles were sampled). For isopods it is known that they are able to accumulate metals during their entire life span. Data on the isopod's life strategy and life-cycle in dynamic ecosystems, such as floodplains, are not available. Probably isopods are not exposed to the metal-polluted soil during flooding events and hide in trees. For the sake of simplicity, their maximum exposure time in the field was estimated to be 140 days, similar to the earthworms.

Field validation

Metal concentrations in the pore water modelled over time and depth using the chemical module BIOCHEM are shown in Figure 4 (based on data of Schröder et al submitted and Chapter 2). To illustrate that flooding events have a major impact on metal concentrations in the pore water, the waterlevel and redox potential are shown as well. The fluctuating metal concentrations are the input for the transfer functions. Since *L. rubellus* is expected to live between 5 and 25 cm below the soil surface, metal levels at these depths representing the average exposure concentration are shown.

To validate the transfer functions, metal concentrations in earthworms were modelled using equation 3 and the parameters given in Table 2 and compared to the measured concentrations as given in Chapter 2. The modelled and measured internal Cd, Pb, Zn and Cu concentrations are shown in Figure 5.

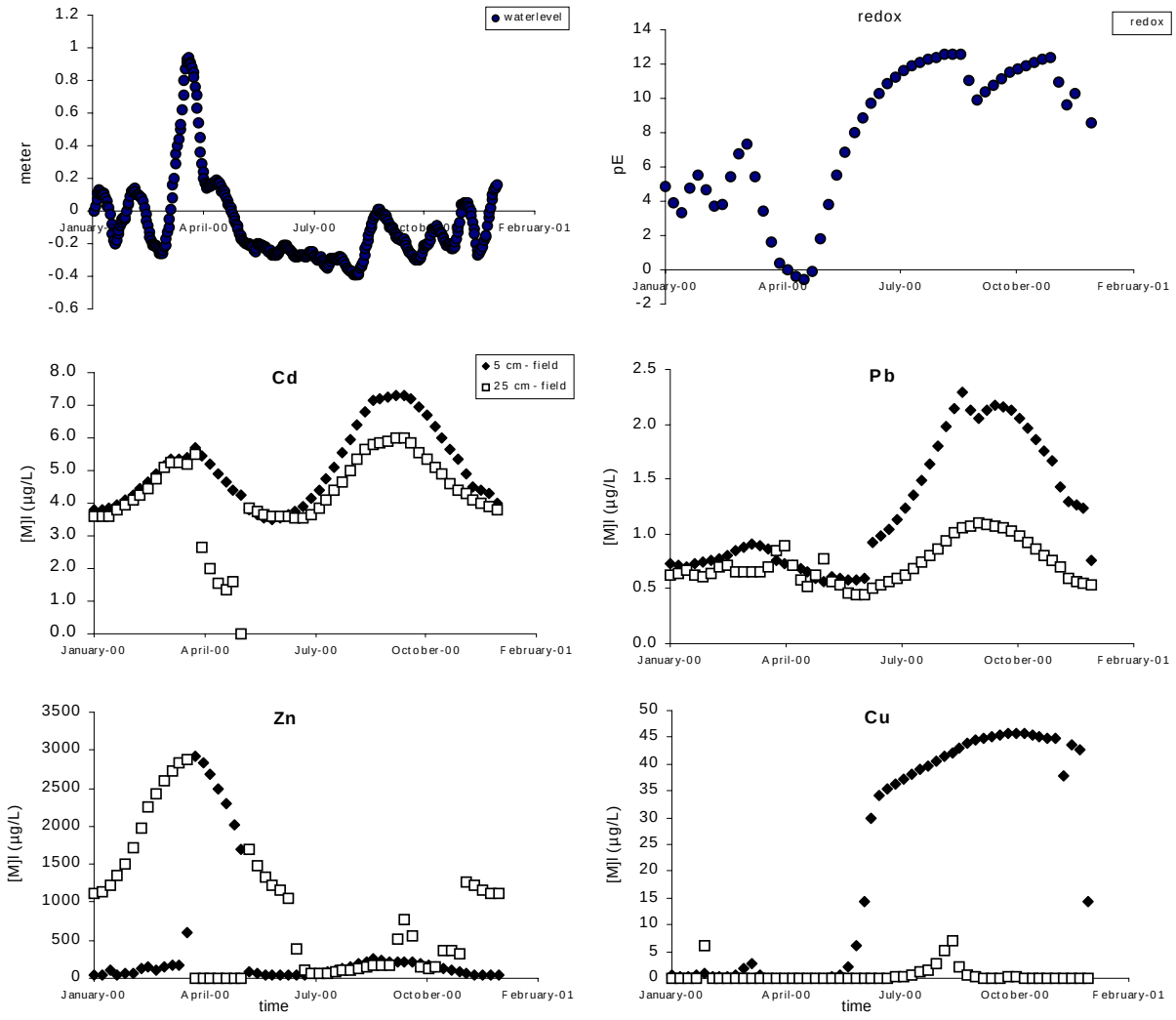

Figure 4: The output of the chemical module BIOCHEM for the floodplain soil M1 as described in Chapter 2. Waterlevel, redox potential (pE), and the pore water concentrations (µg/L) of Cd, Pb, Zn and Cu are displayed. Solid symbols represent the metal concentration 5 cm below the soil surface and the open symbols are metal concentrations at a depth of 25 cm.

The internal metal concentrations in earthworms varied with age, and therefore the exposure time of the earthworms. The large biological variation in measured Cd concentrations in February (Figure 5) can be explained by this parameter due to flooding, just like the slight decrease in modelled Cd concentrations of earthworms in May. Modelled and measured Cd concentrations in earthworms were of a similar order of magnitude. Also for Pb the modelled and measured internal concentrations were of the same order of magnitude. However, the fluctuations in modelled internal Pb levels over time showed a pattern opposite to the measured Pb concentrations. Pb concentration in earthworms was modelled to be higher in the relatively dry months, which resulted from the linear relationship between Pb levels in the pore water and Pb accumulation. The measured Pb concentrations in earthworms were slightly higher and showed a higher biological variance when the water level was high, most likely caused by shorter exposure of (young) earthworms (and higher variance between individuals).

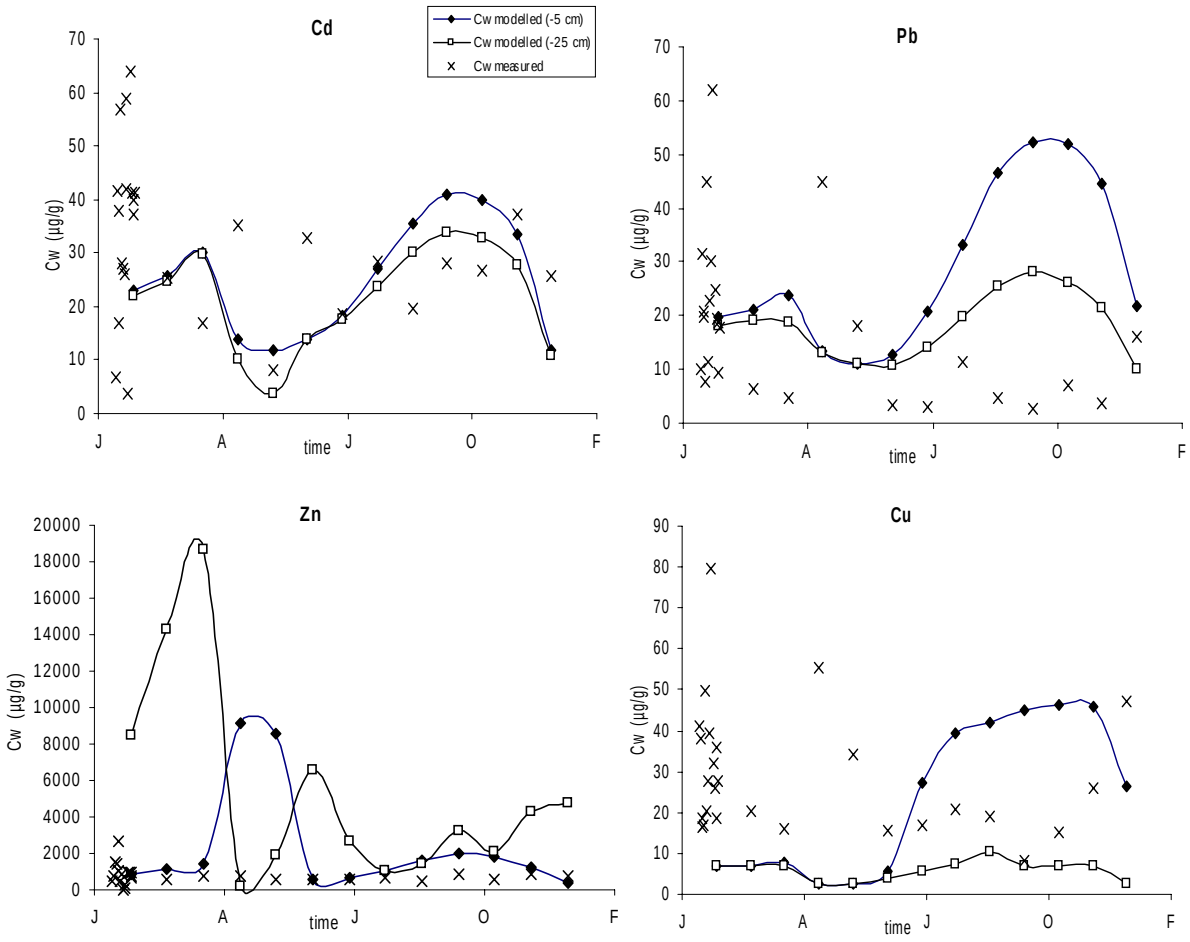

Figure 5: Cd, Pb, Zn and Cu concentrations (µg/g) in the earthworm *Lumbricus rubellus* modelled using the chemical module BIOCHEM and the transfer functions described in this chapter at 5 (solid symbols) and 25 (open symbols) cm below the soil surface and the measured metal concentrations (crosses) taken from Chapter 2.

Modelled Zn concentrations fluctuated largely with the metal concentrations in the pore water during the annual cycle. Measured Zn concentrations were almost constant over the year, of the same order of magnitude as the Zn levels modelled for earthworms in relatively dry months using the pore water concentrations at a depth of 5 cm below the soil surface. For Cu, modelled and measured Cu concentrations were in agreement with each other. Earthworms exposed to the soil at a depth of 25 cm were modelled to have higher accumulation when water level was lower and, as a result, pE was higher.

This validation exercise shows that transfer functions based on physiological partitioning of metals in earthworms coupled to speciation in the soil may describe the general trends in residues of metals in earthworms. The general aspects of the model (internal and external partitioning) are supported, because it is clear that residue dynamics cannot be described by equilibrium partitioning. On the other hand, considerable noise still remains, the source of which remains unknown. More extensive monitoring data are needed to filter biological and chemical meaningful trends from noise and to allow for a validation of the transfer function.

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Summary
Samenvatting

Summary

Metal bioaccumulation kinetics in soil invertebrates in relation to availability and physiology

Scope

Our country deals with a lot of water. Without dikes and dunes, two-third of the country would be flooded. In this context, Napoleon once stated that our country was nothing more than a silty delta of the big rivers crossing his empire. The land bordering the rivers consists of floodplains that serve as water retention basins. Due to the dynamic character of rivers, floodplains consist of variable ecosystems with an array of aquatic and terrestrial gradients.

Water in the rivers was heavily polluted by industrialisation especially in the 1970s - 1980s. These pollution levels are reflected especially in floodplain soils where sedimentation occurs. The soil acts as a sink for metals and other contaminants deposited by the water. Due to the political and society awareness on water quality of rivers, contamination levels have decreased considerably in the late 1980s. However, pollution history still is reflected in the sedimentation layers of the floodplain soils. Therefore, a unique ecosystem nowadays still struggles with high metal levels in the soil (Chapter 2). Rijkswaterstaat initiated the question whether the impact of this pollution results in potential ecological risks at present and/or in the future. This thesis aims at relating metal levels in soil to uptake in soil organisms, accounting also for the way organisms deal with the metals accumulated (Chapter 1).

Earthworms and isopods are selected as test organisms because of their occurrence in floodplain systems, their ability to accumulate metals to a high degree and their physiological differences. Earthworms represent organisms that are in close contact with the pore water of soil, whereas isopods are often found on the soil and in litter layers. Earthworms have a highly water-permeable epidermis, whereas isopods have a calcium-rich exoskeleton that is less water-permeable. *Lumbricus rubellus* is a red pigmented, epigeic earthworm species, which inhabits the top soil layers and litter layers. *Aporrectodea caliginosa* is a weak pigmented earthworm species, inhabiting the mineral soil layers (10-20 cm). The isopod *Porcellio scaber* inhabits, like the earthworm *L. rubellus*, mainly the litter layers on top of the soil.

Metal uptake routes in earthworms

Metals in soil occur in many different species. Not all species are available to organisms in the same extent. Whether uptake in organisms directly or indirectly can be related to metal speciation is dependent on the uptake route. Metals taken up via the skin are directly dependent on the metal speciation in the soil, metals taken up via the digestive tract are influenced by the gut conditions of the organism. The relative contribution of the oral and dermal uptake route was examined by blocking the earthworm's prostomium (mouth) using medical glue and perform bioaccumulation experiments (Chapter 3). Equal metal uptake

was found by sealed and unsealed earthworms exposed to an inert sand matrix continuously flushed with contaminated water. Therefore, pore water uptake via ingestion contributes little to metal accumulation. Sealed and unsealed earthworms were exposed to floodplain soil, and again the dermal route showed to be of most importance for metal accumulation. Cu and Pb accumulation by earthworms exposed to field soil could be attributed to the dermal route for 100%. Internal Cd and Zn concentrations were determined to be 0-17% and 21-30%, respectively due to ingestion.

Uptake and elimination kinetics

Uptake and elimination kinetics of Zn and Cd in the earthworm *Lumbricus rubellus* were determined using radioisotopes (Chapter 4). Radio-isotopes allow for non-destructive measurements in time and allow flux measurements of elements under homeostatic control. Uptake rate constants for Cd were a factor of 2 lower than for Zn. Two distinct compartments could be identified in the earthworm, with different affinities for Zn and Cd. The first compartment is thought to represent the pool of loosely-bound metals (C1), whereas the second one represents a tightly bound storage fraction (C2). The transfer from C1 to C2 is a factor of 10 higher for Cd compared to Zn. The physiological explanation is that Zn is an essential element, and therefore requires high turnover rates as found in C1. Cd is a non-essential element retrieved predominantly from the C2 in which it is tightly bound. Elimination rate constants of Cd and Zn did not differ significantly, which reflect the similarity of elimination mechanisms for both metals. Elimination rate in the first three days after transfer to non-treated soil was high and driven by the C1, later elimination was slow and driven by C2.

The addition of food as a metal source did not affect metal uptake from soil by the earthworm. Because metals are predominantly taken up via the pore water in the soil, replenishment of metal concentrations in the pore water from solid soil particles is important. Therefore, bioavailability cannot be seen as a static equilibrium, but should be based on the dynamics between metal availability in soil and bioaccumulation kinetics.

An accumulation kinetics study using radio-isotopes was also performed with the isopod *Porcellio scaber*, focusing on metal uptake from soil and food (Chapter 5). Metal uptake by the isopod could be described using a one-compartment model, followed by internal distribution of the metal over a reactive and an inert compartment. This partitioning over a reactive and inert compartment resulted in a description of elimination using a two-compartment model. The actual mechanism of bioaccumulation is likely to be more complicated than described by this model, however the simplified model fitted the data accurately.

Comparison of metal accumulation between the two organisms

Earthworms and isopods were exposed to the same floodplain soil in Chapters 4 and 5. When comparing total accumulation of Cd, earthworms accumulate up to 17% more from soil than isopods. After 18 days of elimination, Cd retained in the earthworm in higher amounts than in

the isopod. For Zn, the uptake by earthworms was also higher compared to the isopod. However, after 18 days of elimination, 10% of the accumulated Zn was still retained in the earthworm, whereas the isopod eliminated only 60% of the accumulated Zn. Furthermore, it should be noted that food is a significant uptake source of metals for isopods, while for earthworms the contribution of this source is negligible.

Metal adsorption and absorption

Bioaccumulation is usually based on a summation of the amounts of metal adsorbed to the body wall and absorbed into the body. Whether a metal is adsorbed or absorbed has a different toxicological impact on the organism. Furthermore, adsorption may affect the rate of metal transport over the membrane. Because metal uptake via the epidermis was shown to be dominant (Chapter 3), the relative distinction between adsorbed and absorbed metals was investigated using radio-isotopes (Chapter 6). After 14 days exposure of earthworms and isopods in ^{109}Cd and ^{65}Zn spiked floodplain soil, cross-sections of the organisms were made. Cd and Zn were localized using autoradiography. Surface adsorption of metals onto the body wall of both organisms was shown to be negligible compared to absorption in the body. Correction for metal adsorption in conventional bioaccumulation studies is therefore not required, and bioassays that do consider adsorption and absorption as a total are justified.

Metal sequestration in an organism

Not only metal localisation in organisms is of toxicological importance, even more important is the sequestration of metals over the body (Chapter 7). Each organism evolved mechanisms to protect sensitive bio-molecules from reactive metal ions. These mechanisms are often based on binding metals to proteins or in granules. The mechanisms differ in metal-binding affinity, are strongly based on biochemical binding and are applicable to many organisms. The biochemical partitioning of metals over the organism's body influences elimination kinetics and therefore the level of accumulation. The internal partitioning of metals is metal and organism specific and is dependent on pre-exposure and tolerance. The metal uptake route might affect the sub-cellular distribution.

Sub-cellular metal distribution in earthworms was studied using different exposure regimes (Chapter 8). Cd was mainly retrieved from the cytosolic fraction. The metals Cu, Zn, Ca, Mg and As were mainly found in the active metabolic required fraction consisting of tissue fragments, cell membranes and intact cells. Metals like Pb and Ni could mainly be isolated in the granular fractions disposed with sulphur, whereas Fe was mainly found in the granules consisting of ferritine. This overall pattern of metal distribution found for earthworms collected from the field was used to obtain a bio-classification. This classification is a pragmatic tool that may be useful for screening purposes from which the biological and toxicological significance of each fraction can be identified.

Turnover kinetics of sub-cellular Cd and Zn were determined in earthworms exposed to spiked and natural metal polluted soils. The Cd concentration in the metabolic required fractions consisting of tissue, cell membranes and intact cells did not differ between both

exposure regimes. The metal concentration bound to the cytosolic fraction increased with increasing exposure. For Zn, the sub-cellular concentrations in the granular and metabolic required pool remained constant at the different exposures. Zn in the earthworm's cytosol increased with exposure. For both metals, the kinetics depends on the exposure concentration. Accumulation rates of the different sub-cellular fractions in earthworms exposed to different soils, simulating differences in land use, did not give clear results probably because the experimental set-up was complex.
The internal metal partitioning based on binding affinity corresponds with the bio-classification and is influenced by the physiology of organisms. Knowledge on internal metal distribution in organisms may increase the predictive power of the Critical Body Residue (CBR) concept to estimate effect levels.

Finally
Metal transfer functions, relating bioaccumulation in soil invertebrates to metal speciation in soil, were formulated based on the research described in this thesis (Chapter 9). The parameters of the transfer functions are quantified in the form of a case-study BIOCHEM, based on a scenario-analysis for organisms inhabiting floodplain systems as described in Chapter 2. The transfer functions are the basis for the development of a pragmatic decision-support model functioning as an assessment tool. This tool integrates scientific results with management options to assist decision makers, landowners and researchers in the judgement of the ecological hazards caused by metals in re-development projects. In Chapter 9, the research of this thesis is discussed in a more general context of the work on metal bioavailability, including the applicability of Free Ion Activity Model (FIAM), Biotic Ligand Model (BLM) and the Critical Body Residue (CBR) concept.

Samenvatting

Bioaccumulatiekinetiek van metalen in ongewervelde bodemorganismen in relatie tot beschikbaarheid en fysiologie.

Aanleiding

Water speelt een grote rol in ons land. Zonder dijken, dammen en duinen zou tweederde van ons land zich onder water bevinden. Het is dan ook niet voor niks dat Napoleon ons land beschreef als zijnde: “niks meer dan een ziltige delta gevormd door de grote rivieren van mijn keizerrijk.” Het land direct rondom de rivieren bestaat uit uiterwaarden, die voor waterberging zorgen in tijden dat het rivierwater hoog staat. Door de dynamiek van het water is dit een gevarieerd ecosysteem, dat bestaat uit vele overgangszones van het aquatische en terrestrische milieu.

Het water in de rivieren is ernstig verontreinigd gedurende de industrialisatie met daarbij als dieptepunt de periode 1970-1980. Deze verontreiniging heeft voornamelijk effect gehad op de uiterwaardbodems, alwaar sedimentatie optreedt door het traag stromende of stilstaande water. Metalen en andere vervuilende stoffen uit het water slaan met het zwevend stof van de rivier neer op de bodem. Door de maatschappelijke bewustwording van het grote publiek is de waterkwaliteit in de grote rivieren de laatste jaren enorm verbeterd. Echter de geschiedenis aan vervuiling blijft weerspiegeld in de sedimentatielagen. Hierdoor kampt een uniek stuk natuurgebied in ons land met te hoge gehalten aan metalen in de bodem (hoofdstuk 2). Of dit schadelijk is voor de flora en fauna in de gebieden, is een vraag die Rijkswaterstaat, de beheerder van deze gebieden, graag wil laten onderzoeken. Het doel van dit proefschrift is om een link te leggen tussen metaalconcentraties in de bodem en de opname door organismen, waarbij ook gekeken wordt naar de wijze waarop organismen omgaan met metalen die geaccumuleerd zijn in het lichaam (hoofdstuk 1).

Als toetsorganismen is gekozen voor regenwormen en pissebedden, omdat deze beiden voorkomen in uiterwaarden, metalen in grote hoeveelheden accumuleren en duidelijk fysiologisch van elkaar verschillen in omgang met metalen en opnameroute. Regenwormen bevinden zich voornamelijk in de bodem en staan daarbij in direct contact met poriewater, terwijl pissebedden meer aan het bodemoppervlak foerageren. Regenwormen hebben een waterdoorlatende epidermis hebben, terwijl pissebedden een calciumrijke endoskelet hebben, welke minder doorlatend is voor water. *Lumbricus rubellus* is een rood gepigmenteerde regenworm die voornamelijk in de bovenste 5 cm van de bodem leeft en foerageert. *Aporrectodea caliginosa* is een bleek gepigmenteerde regenworm die zich voornamelijk bevindt in de minerale grond (10-20 cm). De pissebed *Porcellio scaber* bevindt zich, net als de regenworm *L. rubellus*, voornamelijk in de strooisellaag van de bodem.

Opnameroute van metalen in regenwormen

Metalen kunnen in verschillende vormen (species) aanwezig zijn in de bodem. Niet alle vormen zijn in dezelfde mate beschikbaar voor opname door het organisme. Of

metaalspeciatie in de bodem direct of indirect bepalend is voor de hoeveelheid metalen die daadwerkelijk opgenomen wordt door organismen, is afhankelijk van de opnameroute. Metalen die door organismen via de huid opgenomen worden, staan in direct contact met de metaalspeciatie in de bodem, terwijl metalen die opgenomen worden via de dermale route te maken krijgen met de spijsverteringscondities in de darmen en dan een andere vorm kunnen aannemen. De relatieve bijdrage van de dermale en orale route is onderzocht door de routes van elkaar te scheiden en vervolgens metaalopname-experimenten uit te voeren (hoofdstuk 3). De scheiding is bewerkstelligd door het prostomium (mond) van de regenworm *Lumbricus rubellus* te blokkeren met medische weefsellijm. Door wormen bloot te stellen aan inert zand, dat doorspoeld werd met metaal verontreinigd rivierwater, kon worden aangetoond dat metaalopname via het oraal innemen van poriewater geen belangrijke bijdrage levert aan de totale metaalopname. Geblokkeerde wormen en niet geblokkeerde wormen, blootgesteld aan natuurlijke verontreinigde uiterwaardgronden, lieten enig verschil zien in metaal accumulatie in de tijd. De bijdrage van de dermale route bleek hierbij het meest van belang voor de totale opname en bedroeg minimaal 70% en maximaal 100% van de totale opname van verschillende metalen.

Opname- en eliminatiekinetiek

De opname- en eliminatiekinetiek van Cd en Zn in de regenworm *Lumbricus rubellus* is onderzocht met behulp van radio-isotopen (hoofdstuk 4). Radio-isotopen kunnen gemakkelijk worden gedetecteerd in organismen, hebben als voordeel dat ze in tijd kunnen worden gevolgd zonder dat de dieren moeten worden opgeofferd en ondervangen vele problemen, zoals hoge initiële concentraties. Opnamesnelheidsconstanten voor Cd waren gemiddeld een factor 2 lager dan voor Zn. Het verschil in Cd en Zn accumulatie werd voornamelijk veroorzaakt door de verdeling van beide metalen over verschillende interne compartimenten; een snel opnemend en weer elimineerbaar compartiment (C1) en een niet of slechts langzaam elimineerbaar compartiment (C2). De transfersnelheid van Cd vanuit C1 naar C2 was een factor 10 hoger dan voor Zn. De fysiologische verklaring is dat Zn een essentieel element is en noodzakelijk is voor veel biologische processen, waardoor er steeds een hoge turnover moet zijn zoals in C1. Cd is schadelijk voor organismen en komt voornamelijk in het opslagcompartiment (C2) terecht, waar het onschadelijk is en geen interacties met belangrijke bio-moleculen kan aangaan. De eliminatiesnelheidsconstanten verschilden nauwelijks voor Cd en Zn, wat duidt op een zelfde principe van eliminatie van het essentiële Zn en het niet-essentiële Cd. In de eerste dagen werd een snelle eliminatie gevonden vanuit C1. Na 2 à 3 dagen is de eliminatie zeer traag tot nihil en wordt dan gestuurd vanuit C2.

Metaalopname door de regenworm vanuit voedsel bleek niet significant bij te dragen aan de totale bioaccumulatie. Doordat metalen voornamelijk via de huid worden opgenomen, speelt nalevering van metalen vanuit de uitwisselbare vaste fasen in de grond naar poriewater een grote rol bij het verklaren van de totale metaalopname in regenwormen. Dit maakt duidelijk dat biobeschikbaarheid van metalen niet kan worden gezien als een statisch evenwicht, maar dat het een dynamisch evenwicht is van de metaal-beschikbaarheid in de grond en de kinetiek van de bioaccumulatie.

Accumulatiestudies met behulp van radio-isotopen zijn eveneens uitgevoerd met de pissebed *Porcellio scaber*, echter hier met name gericht op bepaling van de accumulatiekinetiek van Cd en Zn vanuit grond en voer (hoofdstuk 5). De metaalopname door de pissebed kon beschreven worden volgens een één-compartimentsmodel, waarna herverdeling van de metalen in het lichaam plaats vond naar een inert compartiment. Vanuit het inerte compartiment vond geen eliminatie plaats, wat resulteert in een eliminatiemodel met tweecompartimenten. Het mechanisme achter deze opname- en eliminatieprocessen is gecompliceerder dan het model doet vermoeden, maar het model is voldoende in staat om het accumulatieproces te beschrijven.
Opnamesnelheidsconstanten vanuit de grond en vanuit het voer waren gelijk voor Cd en Zn. Tevens waren de opnamesnelheidsconstanten vanuit grond en voer additief. De hoeveelheid metalen opgenomen vanuit het voer of de grond is hiermee afhankelijk van de concentraties in grond en voer. Wanneer verondersteld wordt dat de concentraties in voer en grond in evenwicht zijn, kan dus worden geconcludeerd dat het relatieve belang van de twee opnameroutes bepaald wordt door de verdelingscoëfficiënt voor de metaaluitwisseling tussen grond en voer.

Vergelijking van de metaalaccumulatie in de twee organismen
De regenwormen en pissebedden zijn in dezelfde grond met dezelfde hoeveelheid aan radio-isotopen blootgesteld. Wanneer alleen gekeken wordt naar de Cd-opname vanuit de grond, dan kan geconcludeerd worden dat de regenworm na 14 dagen blootstelling 17% meer accumuleert dan de pissebed. Na 18 dagen eliminatie blijft er in de regenworm meer Cd achter dan in de pissebed. Netto accumulatie in de regenworm vanuit de grond is dan ook hoger. Voor Zn is de opname in de regenworm eveneens hoger dan in de pissebed. De regenworm kan het grootste deel van het opgenomen Zn echter elimineren tot maximaal 10% van de opgenomen hoeveelheid, terwijl de pissebed 60% van het opgenomen Zn na 18 dagen elimineren nog in zijn lichaam heeft. Bij deze vergelijkingen moet rekening gehouden worden met het feit dat de pissebed naast grond ook metalen kan opnemen vanuit het voer, terwijl dat voor de regenworm niet tot nauwelijks het geval is.

Adsorptie en absorptie van metalen
In studies waarin interne metaal concentraties worden bepaald, is bioaccumulatie veelal gebaseerd op de som van metalen geabsorbeerd in en geadsorbeerd aan een organisme. Of een metaal geadsorbeerd of geabsorbeerd is, kan grote invloed hebben op de toxiciteit voor het organisme. Het kan ook invloed hebben op de opnamesnelheid van metalen getransporteerd over de membraan. Daar metaalopname via de epidermis dominant bleek te zijn (hoofdstuk 3), is geprobeerd om adsorptie en absorptie te scheiden (hoofdstuk 6). Na 14 dagen blootstelling van regenwormen en pissebedden in een uiterwaardgrond verrijkt met Cd en Zn radio-isotopen, zijn dwarsdoorsnedes (coupes) gemaakt van deze organismen. Met behulp van autoradiografie zijn Cd en Zn gelokaliseerd in deze coupes. Bij het uitlezen van de autoradiografie beelden is specifiek gelet op de regio rond de epidermis, met name of de

metalen zich in of buiten het lichaam bevinden. Adsorptie van metaal aan de epidermis bleek verwaarloosbaar ten opzichte van de absorptie van metalen. Verdiscontering voor adsorptie bij kwantificering van bioaccumulatie is dus niet nodig, zodat er geen aanpassing van de huidige opzet van bioaccumulatiestudies noodzakelijk is.

Verdeling van metalen in een organisme

Niet alleen de lokalisatie van metalen in het lichaam is van toxicologisch belang, nog belangrijker is de vorm waarin de metalen voorkomen in het lichaam (hoofdstuk 7). Elk organisme heeft mechanismen ontwikkeld voor het beschermen van gevoelige bio-moleculen tegen een te veel aan reactieve metaalionen. Deze mechanismen zijn veelal gebaseerd op het binden van metalen aan proteïnen of het invangen van metalen in granulaten. Deze mechanismen verschillen onderling in metaalbindingsaffiniteit, maar zijn toepasbaar op alle diersoorten. De biochemische verdeling van metalen over het lichaam heeft directe consequenties voor de eliminatiekinetiek en daarmee ook voor het uiteindelijke accumulatieniveau dat wordt bereikt. De interne verdeling van metalen over het lichaam is metaal- en organisme-specifiek en afhankelijk van de tolerantie en adaptatie van het organisme. De route van metaalopname kan een rol spelen bij de sub-cellulaire verdeling van metalen in het lichaam.

De sub-cellulaire verdeling van metalen in regenwormen is bestudeerd onder verschillende blootstellingsomstandigheden (hoofdstuk 8). Cd bevindt zich voornamelijk in het cytosol. De metalen Cu, Zn, Ca, Mg en As komen veelal voor in de actieve metabolismefractie, bestaande uit o.a. weefsels en celmembranen en in de cytosol fractie. Metalen zoals Pb en Ni, die voornamelijk te vinden zijn in de granula-fractie, gebonden aan zouten en cysteine-afbraakproducten. Fe is voornamelijk terug te vinden in de granula-fracties, gebonden aan ferritine. Op basis van deze interne verdeling van metalen in regenwormen uit het veld is een bio-classificatie gemaakt. Wanneer de metaalverdeling over deze bio-classificatie sterk afwijkt, kan dat een indicatie zijn voor stress.

De opname- en eliminatiekinetiek van metalen in de verschillende fracties voor metalen zijn bepaald bij regenwormen, die blootgesteld zijn aan metaal-verrijkte en natuurlijk verontreinigde veldgronden. Voor Cd was te zien dat de concentratie in de metabolisch actieve fractie - bestaande uit weefsels en celmembranen - in organismen blootgesteld aan metaal-verrijkte en natuurlijk verontreinigde gronden gelijk is. De Cd-concentratie in het cytosol stijgt met de blootstellingsconcentratie. Deze fractie heeft dan ook de beschermende functie om het te veel aan Cd te detoxificeren. De turnover-kinetiek van de cytosol fractie is het hoogste vergeleken met de andere interne fracties. Voor Zn bleef de concentratie min of meer constant in de metabolisch actieve fractie en de granula fractie in de regenwormen blootgesteld aan gespikte en natuurlijk verontreinigde gronden. De cytosol fractie gaf voor Zn net als voor Cd de grootste turnoversnelheid. Voor beide metalen geldt dat de kinetiek van de sub-cellulaire fracties afhankelijk is aan de blootstellingsconcentratie. Accumulatiesnelheden van de verschillende fracties bij blootstelling van regenwormen gedurende veranderend landgebruik, welke gesimuleerd werd door het blootstellen van veldwormen aan een andere veldgrond, gaf geen eenduidige resultaten.

De interne metaalverdeling is gebaseerd op bindingsaffiniteit, overeenkomstig met de bio-classificatie, en wordt beïnvloed door de fysiologie van het organisme. Kennis over deze interne verdeling kan bijdragen aan een nauwkeurigere inschatting van effecten dan mogelijk is op basis van totale interne lichaamsconcentraties.

Tenslotte

Op basis van de onderzoeken beschreven in dit proefschrift zijn transferfuncties voor metalen geformuleerd die de bioaccumulatie in organismen vanuit de bodem beschrijven (hoofdstuk 9). De kwantificering van de transferfuncties is uitgewerkt aan de hand van een casus in de Nederlandse uiterwaardgebieden rond de Nieuwe Merwede en Waal (hoofdstuk 2 en 9). Deze transferfuncties dienen als de wetenschappelijke basis voor een Beslissings Ondersteunend Systeem (BOS), dat ingezet kan worden bij het inschatten van de effecten van metalen bij (her)inrichting van een gebied. De doelgroep voor dit BOS zijn gebiedsbeheerders, planvormers en ontwikkelaars, adviseurs en onderzoekers. De onderzoeken worden eveneens bediscussieerd in het licht van de huidige wetenschappelijke ontwikkelingen rondom het formuleren van opname- en effectmodellen, zoals Free Ion Activity Model (FIAM), Biotic Ligand Model (BLM) en Critical Body Residue (CBR) concept.

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Zowel Bie als mijzelf zijn namelijk
niet aan de Alkohol, niet aan de
Drugs, maar reeds jaren Aan Het Werk.
Wat een hele erge verslaving is.
Aan Het Werk.
Daar kom je namelijk nooit meer van af
en je hebt steeds méér nodig.
Op het laatst begin je al om 8 uur ’s ochtends.
En dan de hele dag door. Vreselijk.
Terwijl het leven zònder werk toch óók heel gezellig kan zijn.

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Martina Vijver Utrecht, jan 2005

Curriculum

Martina (Martha Gerdina) Vijver werd geboren op 28 april 1975 in IJlst (Friesland). In 1994 behaalde zij het VWO-diploma aan het Bogerman College in Sneek. Datzelfde jaar begon ze met een HBO-studie milieuchemie aan de Rijkshogeschool IJselland te Deventer. De studie is afgerond met twee stages, de eerste aan de University of Warsawa, Poland. De tweede stage op het veldstation Jacobahaven van het Rijksinstituut voor Kust en Zee (RIKZ) te Kamperland. Na afronding heeft ze voor korte tijd gewerkt als data analist industriële effluenten bij Rijkswaterstaat Directie Noord-Nederland te Leeuwarden. Vervolgens is ze gaan werken bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven op het Laboratorium voor Ecotoxicologie in de groep van Dr. W. Peijnenburg. Via een consortium van Rijkshogeschool IJselland en de University of Greenwich, UK, Dept. Environmental Science heeft ze in 2000 haar Master of Science by research diploma *cum laude* behaald. Korte tijd heeft ze via een uitzendbureau gewerkt op het Rijksinstituut voor Integraal Zoetwaterbeheer en Afvalwaterbehandeling (RIZA) te Lelystad. In 2000 is ze gaan promoveren op de Vrije Universiteit Amsterdam, afdeling dieroecologie bij prof.dr. N.M. van Straalen en dr.ir. C.A.M. van Gestel. Het promotieonderzoek was in opdracht van dr.ir. J.P.M. Vink werkzaam op het RIZA in Lelystad. Het promotieonderzoek heeft geleid tot dit proefschrift, welke is afgerond in 2004 met daarbij de certificering van de onderzoeksschool SENSE.

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